# WEST Search History

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| ☐ | L42 | L35 and 236G | 0 |
| ☐ | L41 | L35 and 235A | 2 |
| ☐ | L40 | L35 and 234V | 0 |
| ☐ | L39 | L38 and 233(proline) | 27555 |
| ☐ | L38 | L35 and 233 | 95 |
| ☐ | L37 | L35 and 233P | 0 |
| ☐ | L36 | L35 and (reduced)adj(complement) | 2 |
| ☐ | L35 | L34 and (complement) | 181 |
| ☐ | L34 | L33 and substitution | 197 |
| ☐ | L33 | L32 and (CH2)adj(domain) | 215 |
| ☐ | L32 | 530/387.3,388.15,388.2,388.22,388.25,388.7.ccls. | 3195 |
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| ☐ | L22 | L20 and 236G | 0 |
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| ☐ | L19 | L18 and 234V | 2 |
| ☐ | L18 | L17 and 233P | 2 |

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| ☐ | L16 | L15 and Fc | 13367 |
| ☐ | L15 | L14 and (heavy)adj(chain) | 18415 |
| ☐ | L14 | (immunoglobulin)same(antibod?) | 41810 |
| ☐ | L13 | L7 and 331S | 3 |
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| ☐ | L11 | L7 and 327G | 2 |
| ☐ | L10 | L9 and 236G | 0 |
| ☐ | L9 | L8 and 233P | 2 |
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| ☐ | L7 | L1 and 233P | 7 |
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| ☐ | L4 | L1 and (altered)adj(effector)adj(function) | 132 |
| ☐ | L3 | L2 and (reduced)adj(complement) | 10 |
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**PII: S0161-5890(97)00112-0**

# AMINO ACID DIFFERENCES IN THE N-TERMINUS OF $C_H2$ INFLUENCE THE RELATIVE ABILITIES OF IgG2 AND IgG3 TO ACTIVATE COMPLEMENT

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**Abstract**—The four human IgG isotypes are highly conserved in amino acid sequence, but show differential ability to activate complement (C'): IgG3 and IgG1 are very active, IgG2 is active under certain conditions, and IgG4 is inactive. Although the second constant domain ($C_H2$) is critical for C' activation, the individual amino acids that confer isotype-specific activity have not been identified. We have generated a series of mutants between IgG2 and IgG3, resulting in the exchange of the four N-terminal and six C-terminal polymorphic residues within $C_H2$. Mutants containing the N-terminus of the $C_H2$ of IgG3 were as effective as wildtype IgG3 in C1q binding, C1 activation and terminal complex (MAC) formation, but had reduced ability to effect C'-mediated lysis. IgG2 and mutants containing the N-terminal portion of the $C_H2$ of IgG2 were reduced compared to IgG3 in activating C1, binding C1q and inducing assembly of the MAC, and were inactive in mediating lysis of target cells. Thus, the amino acid sequence differences in the N-terminus of $C_H2$ play a critical role in determining the relative abilities of IgG2 and IgG3 to bind C1q and activate the C' cascade although additional residues of $C_H2$ must be involved in mediating optimal target cells lysis. The sequence of the N-terminus of $C_H2$ was less critical in determining C4 and C3 binding. Characterization of domain exchange mutants suggests that intermediate steps may be partly dependent on domains other than $C_H2$. IgGs that do not direct target cell lysis nevertheless activate intermediate steps in the pathway, which may contribute to immune complex-associated disorders. © 1997 Elsevier Science Ltd. All rights reserved.

*Key words:* chimeric antibody, immunoglobulin, Ag binding, complement, C'

## INTRODUCTION

A major function of immunoglobulins (Igs) is to induce clearance of pathogenic antigens by activating the complement (C') system. Classical pathway activation by immune complexes (ICs) involves a series of reactions that culminate in formation by the terminal C' complex (MAC) of a lytic pore on a target cell surface. Although lysis results from activation of the entire C' cascade, additional effects result from activation of the intermediate components C4, C3 and C5, which liberate fluid phase products (C3a, C4a and C5a) that induce inflammatory responses (Kohl and Bitter-Suermann, 1993). The solid phase product C3b and its degradation products iC3b and C3dg also play important roles in marking ICs for clearance via C' receptors CR1 (Ross and Medof,

1985) and CR3 (Law, 1988) on erythrocytes and macrophages, respectively. Therefore, it is important to understand how Igs activate not only cell lysis, but also individual steps in the C' cascade. Indeed, differences in ability to activate intermediate components may result in differences in lytic ability of an IgG.

In humans, only the IgG and IgM isotypes are effective immunoglobulin activators of the classical C' pathway. Within the IgG subclass, the isotypes IgG1, IgG2, IgG3 and IgG4 are highly conserved in amino acid sequence (Kabat *et al.*, 1991), but differ in their abilities to direct C'-mediated cell lysis. Chimeric anti- 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) IgGs and anti-1-dimethylamino-naphthalene-5-sulfonyl (DNS) IgGs generally show a hierarchy of IgG3 > IgG1 > IgG2 ≫ IgG4 in their abilities to induce C'-mediated lysis (Garred *et al.*, 1989; Lucisano and Lachmann, 1991; Michaelsen *et al.*, 1991; Dangl *et al.*, 1988). However, Bindon *et al.* (1990) reported that anti-NIP IgG1 is more effective at inducing C'-mediated lysis than IgG3 and this effect was shown later to be due to enhanced activation of C4. These differences may be dependent on antigen (Ag) density and Ab concentration (Garred *et al.*, 1989; Lucisano and Lachmann, 1991; Michaelsen *et al.*, 1991).

Structural differences among the human IgG constant

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*Abbreviations:* IC, immune complex, H, immunoglobulin heavy chain, L, immunoglobulin light chain, DNS, 1-dimethylamino-naphthalene-5-sulfonyl-, HBS, HEPES-buffered saline, MAC, terminal C' complex.

domains are thought to be responsible for the isotypic differences in C' activation. The hinge regions show more sequence diversity than the other domains and previous studies correlated hinge flexibility with C' activation (Dangl *et al.*, 1988; Isenman *et al.*, 1975). More recent studies, however, have shown that the hinge flexibility *per se* does not determine isotype-specific differences in human IgG. An IgG4 with the long and flexible hinge of IgG3 is unable to activate C' (Tan *et al.*, 1991) and IgG3s with shortened hinges show similar or increased ability to activate C' compared to wildtype IgG3 (Tan *et al.*, 1991; Michaelsen *et al.*, 1990).

An important role for the second constant domain ($C_H2$) has been supported by numerous studies. Initially, both Facb (IgG depleted of $C_H3$) and Cgamma2 fragments were shown to be active in C' fixation, whereas Cgamma3 fragments were inactive (Colomb and Porter, 1975; Yasmeen *et al.*, 1976). In addition, aglycosylated IgGs lacking the normal $C_H2$-associated carbohydrate are deficient in their ability to activate C' (Tao and Morrison, 1989; Leatherbarrow *et al.*, 1985) while IgG2 containing the $C_H2$ of IgG3 directed C'-mediated lysis as well as wildtype IgG3 (Tao *et al.*, 1991). However, our laboratory has shown recently that all four human isotypes engineered to form polymeric IgGs are able to bind C1q and direct C'-mediated lysis (Smith *et al.*, 1995) suggesting that sites within $C_H2$ required for C' activation may be present in IgG2 and IgG4 but are of low affinity or inaccessible in the monomers.

Although there are a limited number of polymorphic residues within the $C_H2$ of the four human isotypes, the amino acids critical for determining isotype-specific C' activation have not been accurately defined. Residues Glu318, Lys320 and Lys322 in $C_H2$ (Duncan and Winter, 1988) were shown to contribute to the C1q binding site, but since all four human IgGs have these residues, other factors must determine the differences. Mutation of Lys276 to Asn results in decreased ability of IgG3 to activate C' but the reciprocal mutation does not increase activity of IgG1 (Tao *et al.*, 1993). The presence of Ser at position 331 in the COOH-terminal portion of $C_H2$ contributes to IgG4's inability to activate C' (Tao *et al.*, 1991) and mutation of Pro331 to Ser results in reduced (IgG3) or lost (IgG1) ability to direct C'-mediated lysis (Tao *et al.*, 1993). Although mutation of Ser331 to Pro confers some C' activation ability to IgG4 (Tao *et al.*, 1993) it does not make IgG4 as active as IgG1 or IgG3 suggesting additional polymorphic residues play a role in IgG4s, deficiency in C' activation.

Taken together, these data suggest that multiple amino acid residues are required for optimal C' activation and that the tertiary or aggregated structure may also be important. In order to further investigate the structural features of $C_H2$ that are critical for C' activation, we have engineered a series of anti-DNS mouse–human chimeric molecules containing IgG2/IgG3 hybrid $C_H2$ domains and have analysed these mutants for their ability not only to direct C' mediated lysis, but also to activate individual steps in the C' cascade. Our data suggest that the N-terminal portion of $C_H2$ is critical for optimal IgG3-like activity and the overall ability of an IgG to activate the entire cascade is dictated by its ability to bind C1q. However, we also show that IgGs that do not direct target cell lysis can activate intermediate steps in the pathway, which may contribute to elimination of antigen or to pathogenesis in inflammatory diseases.

## MATERIALS AND METHODS

### Construction of IgG mutants

Chimeric IgG genes encoding mouse variable regions specific for the hapten dansyl (DNS) and human constant regions for wildtype IgGs (Shin and Morrison, 1989) and domain-exchanged IgG2 and IgG3 mutants (Tao *et al.*, 1991) were constructed previously. A convenient *Eco*81 I restriction site in $C_H2$ was used to create the hybrid-$C_H2$ genes 2-2-2/3-2, 2-2-3/2-2 and 3-3-3/23, where each number refers to the isotype of the domain according to the scheme $C_H1$–Hinge–$C_H2$–$C_H3$. Mutant IgGs were initially constructed in pBR vectors that contain the IgG heavy chain constant regions as Sal I-BamH I cassettes. For expression in myeloma cells, Sal I-BamH I fragments containing the entire mutant constant regions were subcloned into the pSV2ΔH*gpt* that contains the anti-DNS $V_H$ derived from the mouse hybridoma 22–44 (Dangl *et al.*, 1988). The vector pSY2ΔH*neo* that expresses the anti-DNS mouse Vκ fused to human Cκ was described previously (Morrison and Oi, 1984).

### Expression and purification of IgGs

The non-Ig-producing myeloma cell line Sp2/0 stably transfected with the mouse–human chimeric κ light chain is available in the laboratory. Cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% bovine calf serum (HyClone Laboratories, Inc., Logan, Utah, U.S.A.), penicillin (10 µg/ml), streptomycin (10 µg/ml) and 50 µM 2-mercaptoethanol. Expression vectors containing the mutant IgG heavy chains were transfected into the light chain-producing myeloma cells by electroporation as described (Shin and Morrison, 1989). Selection medium was IMDM containing 10% FCS, 3.8 µg/ml hypoxanthine, 63 µg/ml xanthine and 1.5 µg/ml mycophenolic acid. For screening, microtiter plates (Immulon, Dynatech Labs, Inc., Chantilly, VA, U.S.A.) were coated with 50 µl of dansyl–bovine serum albumin conjugate (DNS–BSA; 10 µg/ml) and blocked with 100 µl of phosphate-buffered saline (PBS; 0.15 M NaCl, 50 mM phosphate, pH 6.8) containing 3% BSA. Transfection supernatants (50 µl) were bound to plates overnight at 4°C and bound IgGs were detected using an anti-human kappa alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO, U.S.A.). Selected clones were expanded and protein production was verified by biosynthetic labeling and SDS–PAGE. IgG proteins were purified from roller bottle culture supernatants by DNS–Sepharose affinity chromatography as described (Tao and Morrison, 1989). Purified proteins were quantified by the bicinchoninic

acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.) and analysed by SDS–PAGE to verify integrity and concentration.

### C'-mediated hemolysis

The abilities of mutant and wildtype IgGs to direct C'-mediated lysis of DNS–BSA-coated sheep red blood cells (SRBC) were measured essentially as described (Tao et al., 1991), except that 10 $CH_{50}$ units of human (normal human serum from healthy donors) or guinea pig C' (Colorado Serum Co., Denver, CO, U.S.A.) were used. IgGs, DNS–BSA-coated, $^{51}$Cr-loaded SRBCs and serum C' were incubated for 45 min in a 37°C water bath. Release of $^{51}$Cr was measured in supernatants following centrifugation of cells. Background lysis (C' alone) and total lysis (SRBC in water) were used to calculate percentage lysis. Human serum was collected in Vacutainer® Brand serum separator tubes (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) and allowed to clot for 30 min at room temperature prior to centrifugation at 4°C. Serum was then preabsorbed at 4°C in two steps by incubation first with an equal volume of packed SRBC and second with 0.1 volumes of DNS–BSA-coated SRBC. All serum was aliquoted and stored at −70°C.

### ELISA for C1q binding

Binding of C' component C1q was performed using a modification of the method described previously (Tao et al., 1993). Microtiter plates (Immulon 2, Dynatech Labs, Inc., Chantilly, VA, U.S.A.) were coated overnight at 4°C with 100 μl DNS–BSA (32:1 DNS:BSA ratio) at either low or high density (0.5 or 10 μg/ml, respectively). Plates were washed six times with PBS and blocked with PBS containing 3% BSA for either 2 hr at room temperature or overnight at 4°C. Antibodies (0 to 2 μg/ml in 100 μl PBS containing 1% BSA) were incubated with Ag-coated plates overnight at 4°C. Plates were then washed three times with PBS and three times with Hepes-buffered saline (HBS) (0.15 M NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 10 mM Hepes, pH 7.4) containing 0.1% Tween-20 (HBST). Human serum (0.125%, 100 μl) diluted in HBS was then added as the C' source and plates were incubated for 1 hr in a 37°C water bath. Plates then were washed three times with HBST and three times with PBS. For detection of bound C1q, 100 μl of goat anti-human C1q (Quidel, Inc., San Diego, CA, U.S.A.) diluted 1:10000 was incubated with plates for 1 hr in a 37°C warm room. Following six washes with PBS, a swine anti-goat alkaline phosphatase conjugate (1:20000 dilution) was added for 1 hr at 37°C. Plates were washed six times with PBS and 100 μl (0.5 mg/ml) p-nitrophenyl phosphate substrate (Sigma Chemical Co.) in diethanolamine, pH 9.8, were added. Color was allowed to develop at room temperature for 30 min to 1 hr. Plates were read at 405 nm using an automated ELISA reader (Dynatech Laboratories, Inc., Chantilly, VA, U.S.A.).

### ELISA for binding of C' components C4, C3 and the C5b–C9 membrane attack complex (MAC)

ELISAs to measure binding of components C4b and C3b were carried out exactly as described for C1q, except that the detecting reagents were goat anti-human C4 diluted 1:10000 and goat anti-human C3 diluted 1:5000 (both from Quidel, Inc.), respectively. The ELISA for the MAC was done with several modifications. First, detection of bound MAC required use of 1% serum as the C' source. Second, the detecting reagent was a mouse monoclonal against the SC5b-9 neoepitope (Quidel, Inc.) diluted 1:10000. The developing reagent was a rabbit anti-mouse alkaline phosphatase conjugate (Sigma Chemical Co.) diluted 1:1000. Controls for these assays were as described above. Also, no binding of C3 or MAC was observed in the presence of EGTA and $Mg^{2+}$, indicating that binding was specifically due to classical pathway activation.

### Binding of mutant and wildtype IgGs to DNS–BSA-coated microtiter plates

The amount of purified IgG bound to the Ag-coated plates used for the C' component ELISAs was determined using a polyclonal anti-human gamma-alkaline phosphatase conjugate (Sigma Chemical Co.), which reacts with all human IgG isotypes. Briefly, IgGs (100 μl) containing concentrations from 0 to 2.0 μg/ml were bound to DNS–BSA-coated plates as described above. Following six washes with PBS, 100 μl of conjugate (diluted 1:10000 in PBS containing 1% BSA) were added and plates were incubated for 1 hr at 37°C. Plates were washed six times with PBS and substrate was added as described above.

### C1 activation

Cleavage products of activated C' component C1 were detected by immunoblotting and chemiluminescence. Briefly, wildtype and mutant IgGs (10 μg) were mixed on ice with the DNS–BSA (1 μg) and normal human serum (2 μl) in a total volume of 100 μl. Control samples contained (1) no IgG or (2) IgG3 in the absence of DNS–BSA. Samples were incubated for 2 hr in a 37°C water bath and the reaction was terminated by addition of 25 μl of SDS–PAGE sample buffer and 1 μl of 2-mercaptoethanol. Samples were boiled for 2 min, incubated at 37°C for 30 min and electrophoresed on a 12.5% Tris–glycine acrylamide gel. The gel was fixed in methanol transfer buffer (192 mM glycine, 25 mM Tris–HCl, pH 8.3, 20% methanol) and proteins were transferred to nitrocellulose (BioBlot-NC, Costar Scientific, Cambridge, MA, U.S.A.) at 4°C (2 hr at 0.75 A followed by overnight at 0.1 A) in a Hoeffer Transphor apparatus (Hoeffer Scientific Instruments, San Francisco, CA, U.S.A.). Blots were incubated for 1 hr at room temperature in blocking buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20 and 5% milk) and then incubated for either 1 hr at room temperature or overnight at 4°C with goat anti-human C1r (Atlantic Anti-

bodies, Incstar Corp., Stillwater, MN, U.S.A.) diluted 1:50 in blocking buffer. Blots were washed 3 × 15 min in blocking buffer and then incubated for 1 hr at room temperature with swine anti-goat-HRP conjugate (Sigma Chemical Co.) diluted 1:20 000 in blocking buffer. Following washes (2 × 15 min in blocking buffer, 1 × 15 min in PBS), blots were incubated with the ECL™ chemiluminescent reagent (Amersham, Buckinghamshire, U.K.) as directed and exposed to X-ray film.

*Data analysis*

Direct lysis assays were performed with duplicates for all data points. ELISAs were performed in triplicate. Data shown are representative of two to four assays. For comparison of data from multiple plates within the same experiment, wildtype IgG3 was included on each plate. ELISA data were normalized to IgG3 binding by setting the maximum IgG3 binding to 100%.

## RESULTS

*Production and purification of proteins*

IgG heavy chains were constructed with hybrid $C_H2$ domains in which four N-terminal (233, 234, 235, 236) and multiple COOH-terminal (276, 291, 296, 309, 327, 339) polymorphic residues were exchanged between IgG2 and IgG3 (Table 1). Schematic diagrams for all mutants utilized in these studies are shown in Fig. 1. The various constant regions were joined to the murine anti-DNS variable region and transfected into a myeloma cell line that stably expresses the anti-DNS light chain. Trans-
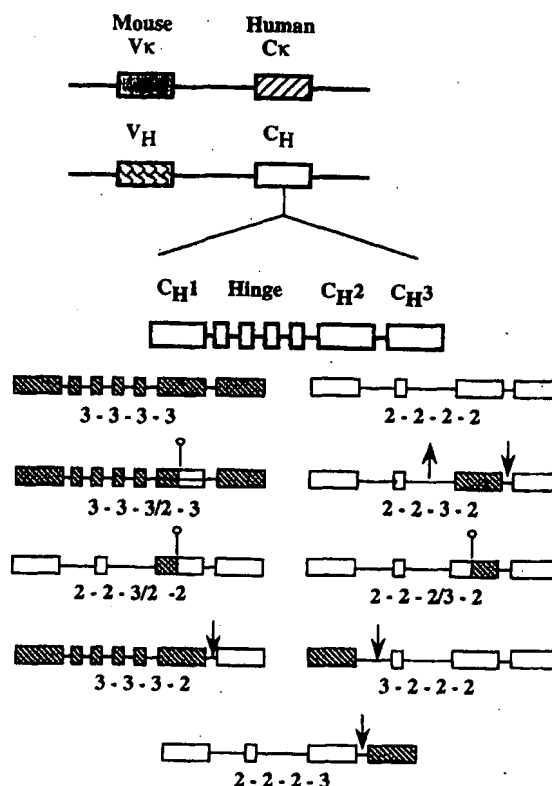


Fig. 1. Schematic diagram of domain-exchanged IgG heavy chains. Up arrows, down arrows and circles represent Sac II site, Pvu I sites and Eco81 I restriction sites, respectively. Sac II and Pvu I were engineered previously using oligonucleotide linkers (Tao *et al.*, 1991). Heavy chain constant regions were joined to the murine anti-DNS variable region. All mutants were given a four digit code according to the scheme $C_H1$–Hinge–$C_H2$–$C_H3$. Numbers represent the isotype of the domain.

Table 1. Polymorphic residues in $C_H2$ among human IgGs 1, 2, 3 and 4

| Residue | IgG1 | IgG2 | IgG3 | IgG4 |
|---------|------|------|------|------|
| 233[a]  | Glu[b] | Pro | Glu | Glu. |
| 234     | Leu  | Val  | Leu  | Phe  |
| 235     | Leu  | Ala  | Leu  | Leu  |
| 236     | Gly  |      | Gly  | Gly  |
| →       |      |      |      |      |
| 268     | His  | His  | His  | Gln  |
| 274     | Lys  | Gln  | Gln  | Gln  |
| 276     | Asn  | Asn  | Lys  | Asn  |
| 291     | Pro  | Pro  | Leu  | Pro  |
| 296     | Tyr  | Phe  | Tyr  | Phe  |
| 300     | Tyr  | Phe  | Phe  | Tyr  |
| 309     | Leu  | Val  | Leu  | Leu  |
| 327     | Ala  | Gly  | Ala  | Gly  |
| 330     | Ala  | Ala  | Ala  | Ser  |
| 331     | Pro  | Pro  | Pro  | Ser  |
| 339     | Ala  | Thr  | Ala  | Thr  |

[a]Position is according to EU numbering.
[b]Sequence is based on Kabat *et al.* (1991) and was confirmed by sequencing of the H chains used in these experiments.
(→) Denotes position for exchange of N- and COOH-terminal portions of $C_H2$.

fectants secreting high levels of IgG were identified by ELISA and the secreted proteins were analysed by ^{35}S-methionine labeling, immunoprecipitation and SDS–PAGE. All of the transfectants produced H and L chains of the expected molecular weights that assembled into $H_2L_2$ tetramers (data not shown). Treatment of cells with tunicamycin and analysis of the immunoprecipitated protein by SDS–PAGE confirmed that the N-linked glycosylation site at Asn 297 in $C_H2$ was utilized (data not shown). The presence of this carbohydrate has been shown to be critical for C′ activation (Tao and Morrison, 1989; Leatherbarrow *et al.*, 1985; Tao *et al.*, 1991).

Purified proteins were then assayed for their ability to effect C′ activation. We employed hemolysis to assay the entire cascade; to assess activation of individual steps (C1q, C4, C3 and MAC) in the C′ pathway, we have employed ELISAs in which IgGs are bound to Ag-coated plates and incubated with human C′. Since Ag concentration and epitope density have been shown to affect an IgG's ability to activate C′ (Garred *et al.*, 1989; Lucisano and Lachmann, 1991; Michaelsen *et al.*, 1991), we have assayed C′ binding using plates coated with both low (0.5 μg/ml) and high (10 μg/ml) concentrations of DNS–BSA.

## Ag binding of $C_H2$ mutant IgGs

In order to be valid, ELISAs for C' component binding require that similar amounts of mutant and wildtype IgGs are bound to the Ag-coated plates. Thus, we first determined the binding of wildtype and mutant IgGs to DNS–BSA-coated microtiter plates. For comparison of mutant and wildtype IgGs, we have expressed data relative to maximal IgG3 binding. Wildtype and mutant IgGs exhibit similar binding to 0.5 μg/ml DNS–BSA-coated microtiter plates in an ELISA with anti-human gamma as the detecting reagent (Fig. 2A). Similarly, IgGs exhibit similar binding to plates coated with 10 μg/ml (Fig. 2B).

## Binding of C1q to $C_H2$ mutant IgGs

C1q activates C1r and C1s by binding to aggregates of two or more IgGs bound to an Ag (Burton, 1985; Hughes and Gardner, 1978; Morgan, 1993). Thus, an IgG's ability to initiate the C' cascade is indicated by its ability to bind C1q. We initially performed controls for each

step in the assay and observed no background binding. Thus, for all subsequent assays we included only the minus-IgG controls. Optimal dilutions for each reagent were also determined. No C1q binding was observed when the assay was performed in the presence of EDTA, an inhibitor of classical pathway activation. Furthermore, we determined that C1q was detected only after activation of C' at 37°C: no binding was observed when IgGs and serum were incubated at 4°C.

At low Ag density, IgG3, IgG2-2-3-2, IgG2-2-3/2-2 and IgG3-3-3/2-3 all bound similar amounts of C1q. In the assay shown, the mutant 2-2-2/3-2 was reduced in its ability to bind C1q and wildtype IgG2 was inactive in C1q binding (Fig. 3A). These two proteins were consistently impaired in their ability to bind C1q; however, the absolute level of C1q binding varied from reduced to none in different assays. We observed similar results when C1q was measured at high Ag density: IgG2 and IgG2-2-2/3-2 were impaired compared to the other proteins in binding C1q (Fig. 3B). No C1q binding was observed
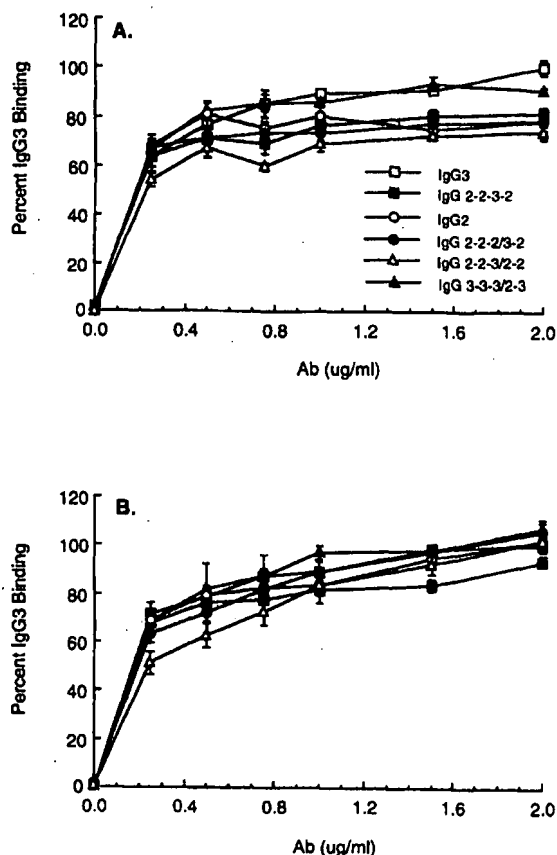


Fig. 2. Binding of IgGs to antigen-coated microtiter plates. Microtiter plates were coated with (A) 0.5 μg/ml DNS–BSA or (B) 10 μg/ml DNS–BSA. Antibodies (0 to 2 μg/ml) were incubated with Ag-coated plates overnight at 4°C. Bound IgGs were detected with an anti-human gamma-alkaline phosphatase conjugate. Data were normalized to the maximum binding observed with IgG3. Each data point represents the mean and standard deviation of triplicate measurements.

Fig. 3. Binding of C1q to wildtype and $C_H2$-mutant IgGs. Microtiter plates were coated with (A) 0.5 μg/ml DNS-BSA or (B) 10 μg/ml DNS–BSA. Antibodies (0 to 2 μg/ml) were incubated with Ag-coated plates overnight at 4°C. Human serum (0.125%) was then added as the C' source. Bound C1q was detected with goat anti-human C1q followed by swine anti-goat alkaline phosphatase conjugate. Data were normalized to the maximum C1q binding by IgG3. Each data point represents the mean and standard deviation of triplicate measurements.
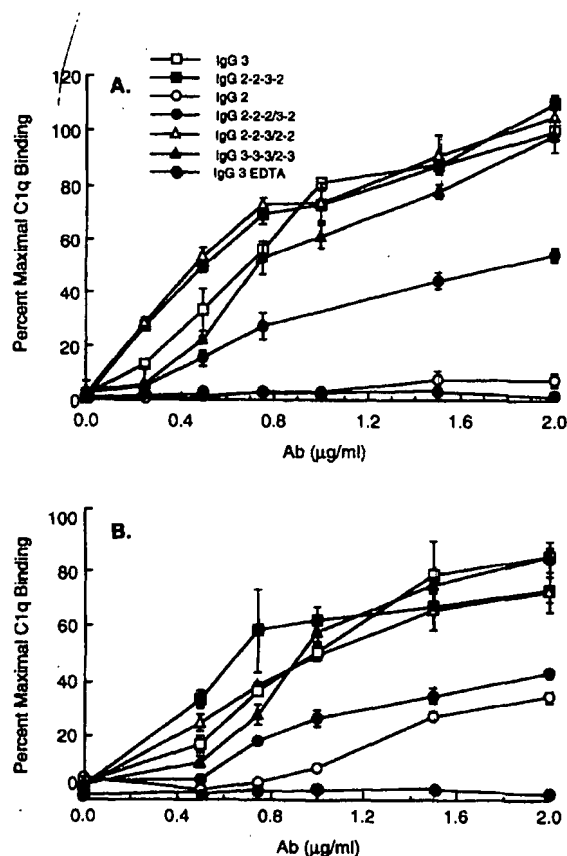
when the assay was performed in the presence of the classical pathway inhibitor EDTA. Thus, the N-terminal portion of $C_H2$ contributes to the differential ability of IgG2 and IgG3 to bind C1q.

### Activation of C1 by wildtype and $C_H2$ mutant IgGs

Binding of C1q represents the first step in the classical pathway of C' activation, but does not indicate whether the serine proteases C1r and C1s were activated. To determine if C1r was activated, we used an anti-C1r immunoblot to detect C1r cleavage products following incubation of IgGs with DNS–BSA and serum. As shown in Fig. 4, the C1r cleavage product of molecular weight 56 000 was detected when C' was incubated with IgG3, IgG2-2-3-2, IgG2-2-3/2-2 and IgG3-3-3/2-3. Little or no protein of molecular weight 56 000 was detected with IgG2 or IgG2-2-2/3-2, or with controls containing no IgG or no DNS–BSA. Thus the ability of the IgGs to activate C1r paralleled their abilities to bind C1q, supporting the observation that the N-terminal portion of $C_H2$ contributes to the differential ability of IgG2 and IgG3 to activate C'.

### Binding of C4b by wildtype and $C_H2$ mutant IgGs

Activated C1s cleaves C4, resulting in binding of C4b to the immune complex. Thus, detection of bound C4b indicates that C4 was activated. At low Ag density (Fig. 5A), equivalent amounts of C4b were bound following activation with either IgG3 or IgG3-3-3/2-3, whereas less C4b was bound with IgGs 2-2-3-2 and 2-2-3/2-2. Therefore, although these proteins bind equivalent amounts of C1q, they differ in their ability to induce C4b binding. Consistent with their reduced ability to bind C1q, IgGs 2 and 2-2-2/3-2 were also reduced in their ability to induce
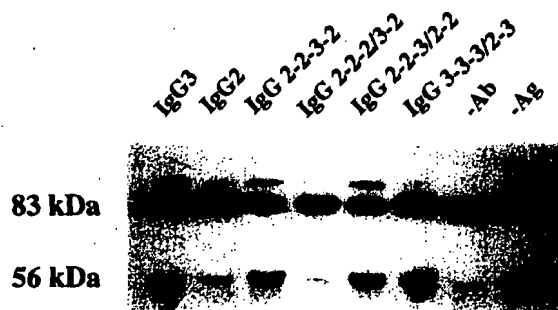


Fig. 4. Activation of C1 by wildtype and $C_H2$-mutant IgGs. IgG2, IgG3, IgG2-2-3-2, IgG2-2-2/3-2, IgG2-2-3/2-2 and IgG3-3-3/2-3 (10 μg) were incubated with DNS–BSA and normal human serum for 2 hr at 37°C. Control samples contained no IgG (−Ab) or contained IgG3 but lacked DNS–BSA (−Ag). Samples were reduced and electrophoresed on a 12.5% Tris–glycine acrylamide gel. Proteins were transferred to nitrocellulose and C1r cleavage products were detected by chemiluminescence following incubation with goat anti-human C1r primary Ab and swine anti-goat-HRP conjugate as secondary Ab. Positions of precursor ($M_r$ 83 000) and cleavage products ($M_r$ 56 000) are indicated.
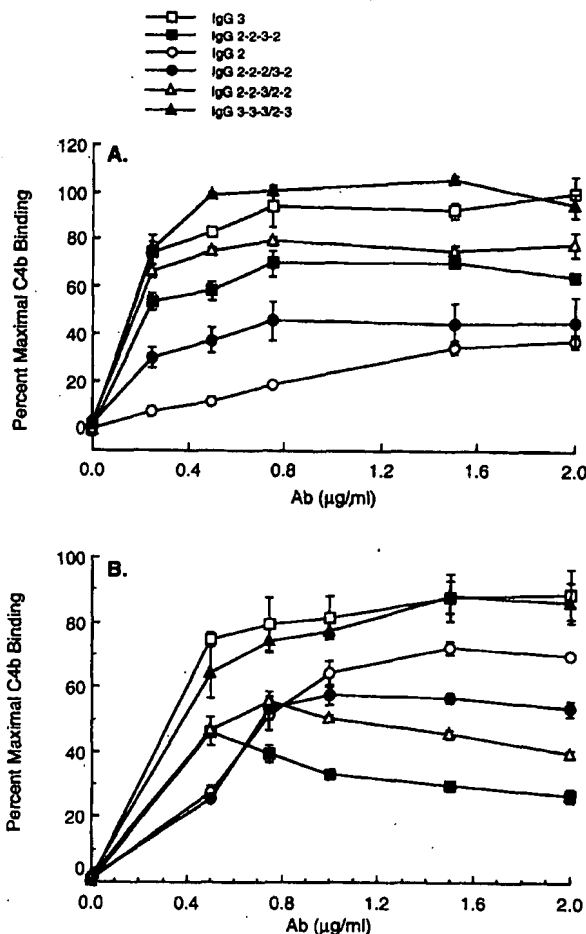


Fig. 5. Induction of C4b binding by wildtype and $C_H2$-mutant IgGs. Microtiter plates were coated with (A) 0.5 μg/ml DNS–BSA or (B) 10 μg/ml DNS–BSA. Antibodies (0 to 2 μg/ml) were incubated with Ag-coated plates overnight at 4°C. Human serum (0.125%) was added as the C' source. Bound C4b was detected with goat anti-human C4 followed by swine anti-goat alkaline phosphatase conjugate. Data were normalized to the maximum C4 binding induced by IgG3. Each data point represents the mean and standard deviation of triplicate measurements.

C4b binding compared to the other proteins and in fact, we sometimes saw no binding of C4b with these proteins. At high Ag density (Fig. 5B) we see a slightly different result. Again IgG3 and IgG3-3-3/2-3 were the most effective at inducing C4b binding and the other proteins were reduced, but the hierarchy of the reduced binders depended on IgG concentration. At low IgG concentration (0–0.5 μg/ml), the hierarchy (IgG2-2-3/2-2 = IgG2-2-3-2 > IgG2-2-2/3-2 = IgG2) was similar to that observed at low Ag density, whereas at higher IgG concentration (0.75–2 μg/ml), the hierarchy was altered (IgG2 > IgG2-2-2/3-2 > IgG2-2-3/2-2 > IgG2-2-3-2). Taken together, these data suggest that both Ag and Ab concentrations can affect the ability of some of the IgGs to activate C4. At low Ag concentration and at high Ag/low Ab, these data indicate an important role for the

N-terminal amino acids in $C_H2$. At high Ag/high Ab, it is possible that other factors influence either C1 activation, C4 cleavage or C4b binding.

### Binding of C3b by wildtype and CH₂ mutant IgGs

Bound C4b binds the component C2a, generating the C3 convertase that cleaves C3 into C3a, which is lost to the fluid phase, and C3b, which is bound to the immune complex. Thus, activation of the C3 convertase is indicated by binding of C3b. At low Ag density we saw more variation in this assay than in the other assays. In general, IgG3 and IgG3-3-3/2-3 were most effective at inducing C3b binding with the other proteins showing varied, but usually reduced, C3b binding (data not shown). At high Ag density (Fig. 6) we consistently observed that IgG3 and IgG3-33/2-3 were more effective activators of C3 than the other proteins. IgG2 showed very low activity at low Ab concentration, but was active at higher Ab concentrations. No binding of C3b was observed in the presence of EGTA and $Mg^{2+}$, indicating that binding was not due to activation of the alternative pathway. Thus, as with C4b binding, we observe that although the N-terminus of $C_H2$ is partially responsible for differences among the mutants, other domains may play a role in activation and binding of C3b.

### Assembly and binding of the membrane attack complex (MAC)

Cleaved C3b binds to the C4b/C2a complex and alters the substrate specificity such that the complex becomes the C5 convertase. In vivo, cleavage of C5 by the con-

vertase results in binding of C5b to membranes and initiates formation of the MAC, which is composed of components C5b, C6, C7, C8 and C9. Since the C5b-9 neoepitope recognized by the detecting antibody used in the assay is exposed only after MAC formation, binding of the MAC indicates both that the C5 convertase was activated and that complex assembly occurred. As shown in Fig. 7, IgGs 2-2-3-2, 3-3-3/2-3 and 2-2-3/2-2 were as effective as wildtype IgG3 at inducing MAC assembly, whereas IgG2 and IgG2-2-2/3-2 were less effective. We also carried out control assays in the presence of EGTA and $Mg^{2+}$. No binding was observed under these conditions, indicating that binding was not due to activation of the alternative pathway (data not shown). These data are consistent with our C1q data and again demonstrate that residues in the N-terminal portion of $C_H2$ contribute to efficient activation of the C' cascade.

### Activation of C'-mediated hemolysis by wildtype and CH₂ mutant IgGs

To assess the overall ability of our IgGs to activate the classical pathway of C', we performed direct lysis assays as described in Materials and Methods. In the presence of human C' (Fig. 8A), IgG2-2-3-2 was as effective as IgG3 in directing lysis of target SRBC. IgGs 3-3-3/2-3 showed similar activity except at high Ab concentration. IgG2-2-3/2-2 also induced lysis, but was reduced compared with IgG3. IgGs 2 and 2-2-2/3-2 showed little activity. In contrast, when we used guinea pig serum (Fig. 8B), IgG2-2-3-2 showed reduced activity and IgG3-3-3/2-3 showed only limited activity compared to IgG3. IgG2 and IgGs 2-2-3/2-2 and 2-2-2/3-2 were essentially inactive in the presence of guinea pig C'. The data obtained with
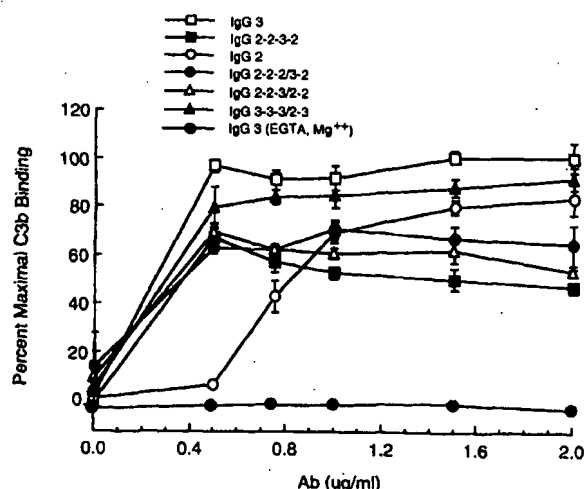


Fig. 6. Induction of C3b binding by wildtype and $C_H2$-mutant IgGs. Microtiter plates were coated with 10 μg/ml DNS–BSA. Antibodies (0 to 2 μg/ml) were incubated with Ag-coated plates overnight at 4°C. Human serum (0.125%) was added as the C' source. Bound C3b was detected with goat anti-human C3 followed by swine anti-goat alkaline phosphatase conjugate. Data were normalized to the maximum C3 binding induced by IgG3. Each data point represents the mean and standard deviation of triplicate measurements.
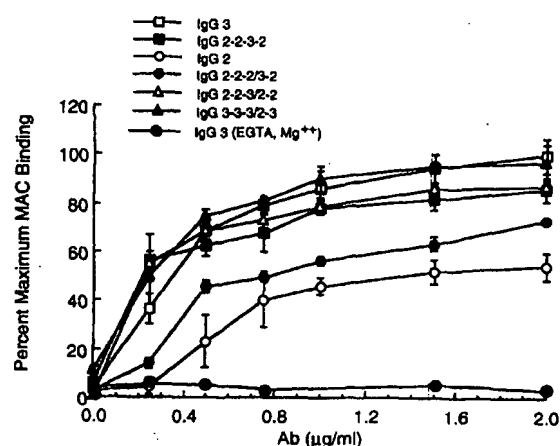
Fig. 7. Induction of MAC assembly and binding. Microtiter plates were coated with 0.5 μg/ml DNS–BSA. Antibodies (0 to 2 μg/ml) were incubated with Ag-coated plates overnight at 4 C. Human serum (1%) was added as the C' source. Bound MAC was detected using the mouse anti-SC5b-9 mAb followed by rabbit anti-mouse alkaline phosphatase conjugate. Data were normalized to the maximum MAC binding induced by IgG3. Each data point represents the mean and standard deviation of triplicate measurements.
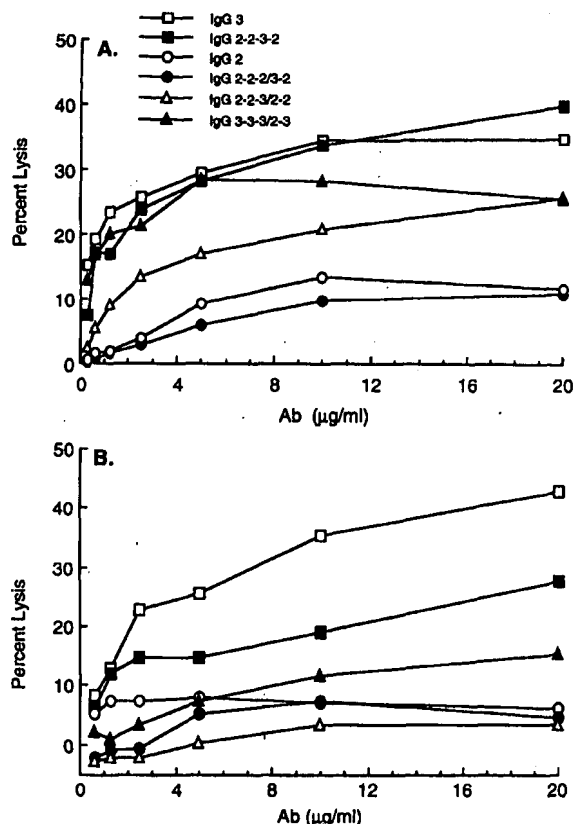
Fig. 8. Complement-mediated hemolysis by wildtype and $C_H2$ mutant IgGs. Ag-coated, $^{51}$Cr-loaded SRBC were lysed with increasing concentrations of IgGs in the presence of (A) human complement (10 $CH_{50}$ units) or (B) guinea pig complement (2 $CH_{50}$ units). Percentage lysis was calculated compared to total lysis of SRBC in water. Each data point represents the mean of duplicate measurements.

human C' support a critical role for the N-terminus of the IgG3 $C_H2$ domain, but indicate that the N-terminus is not sufficient to confer IgG3-like lysis activity, since IgGs 3-3-3/2-3 and 2-2-3/2-2 were not as active as IgG2-2-3-2 and wildtype IgG3.

*Activation of C' by domain-exchanged mutant IgGs*

The data presented above demonstrated that IgGs 2-2-3-2 and 2-2-3/2-2 bound C1q as effectively as IgG3 and 3-3-3/2-3, but showed reduced ability to induce binding of components C4 and C3. The other IgG2 mutants, as well as IgG2, also showed reduced C4 and C3 binding compared to IgG3. This observation suggests that IgG3 domains other than $C_H2$ may play a role in activation or binding of these components. To gain further understanding of this issue, we analysed the following domain-exchanged mutants: IgG3-3-2-2, IgG3-3-3-2, IgG2-2-2-3, IgG3-2-2-2. The ability of these IgGs to activate human C' was assayed at low Ag density. As shown in Fig. 9A, binding to Ag-coated plates was similar for IgG3 and IgG3-3-3-2, reduced slightly for IgG2 and IgG2-2-2-3 and greatly reduced for IgG3-2-2-2. C1q binding was

maximal with wildtype IgG3, slightly reduced with 3-3-3-2 and significantly reduced with IgG2 (Fig. 9B). Of the mutants with the IgG2 $C_H2$, IgG2-2-2-3 showed slight binding at the highest Ab concentration while IgG3-2-2-2 was unable to bind C1q. Similarly, C4 binding was induced by IgG3 and IgG3-3-3-2, but not IgG2, IgG2-2-2-3 or IgG3-2-2-2 (Fig. 9C). We observed different results with C3 (Fig. 9D) and the MAC (Fig. 9E). While IgG3 and IgG3-3-3-2 were most active and IgG3-2-2-2 remained inactive, IgG2 and IgG2-2-2-3 now showed activity. This result was surprising since IgG2-2-2-3 showed no C1q binding or C4 activation and suggested that IgG2-2-2-3 might activate the alternative pathway. However, in the presence of EGTA and $Mg^{2+}$, none of the IgGs induced C3 binding, demonstrating that alternative pathway activation was not responsible for the C3 binding observed with IgG2-2-2-3 (data shown only for IgG2-2-2-3). Although IgG2-2-2-3 activates and binds C3 and generates the MAC, neither it nor IgG3-2-2-2 or IgG3-3-2-2 is effective in C'-mediated lysis (Fig. 9F). Only wildtype IgG3 and IgG3-3-3-2 were effective in directing lysis of target cells.

## DISCUSSION

Our data indicate that amino acid sequence differences in the N-terminal portion of $C_H2$ are important in determining the relative abilities of IgG2 and IgG3 to activate the C' cascade. IgG2 differs from IgG3 at four positions in the lower hinge (Table 1). In our assays, all of the IgGs containing residues Glu233, Leu234, Leu235 and Gly236 (IgG3, IgG 3-3-3/2-3, 2-2-3-2, 2-23/2-2) were comparable to wildtype IgG3 in binding C1q, activating C1 and inducing formation of MAC. However, IgGs 3-3-3/2-3 and 2-2-3/2-2 showed reduced ability to mediate target cell lysis in the presence of human C' compared to IgG3 and IgG2-2-3-2 suggesting that the COOH-terminal portion of $C_H2$ residues contributes to the optimal activity of IgG3. This region of $C_H2$ contains six amino acids (276, 291, 296, 309, 327 and 339) that are polymorphic between IgG2 and IgG3 (see Table 1). Position 276 (Lys in IgG3, Asn in IgG2, IgG1 and IgG4), has been implicated previously for a role in C' activation. Mutation of Lys276 to Asn caused a reduction in IgG1's ability to direct C'-mediated lysis, but the reciprocal mutation (Asn276 to Lys) did not improve IgG1's activity (Tao *et al.*, 1993). Positions 291 (Pro in IgGs 1, 2 and 4, Leu in IgG3) and 296 (Phe in IgGs 2 and 4, Tyr in IgGs 1 and 3) have also been studied previously. Mutation of Pro291 to Leu failed to confer optimal lysis activity to IgG1 and mutation of Phe296 to Tyr failed to activate IgG4 (Tao *et al.*, 1993). However, it is conceivable that these mutations could have an effect in the context of the IgG3/IgG2 hybrid $C_H2$ domain.

The ability of an IgG to non-covalently bind C1q was not directly correlated with its ability to induce covalent binding of C4b and C3b. For example, IgGs 2-2-3-2 and 2-2-3/2-2 were similar to IgG3 in their ability to bind C1q, but showed reduced ability to induce covalent binding of C4b and C3b. This result could reflect either impaired
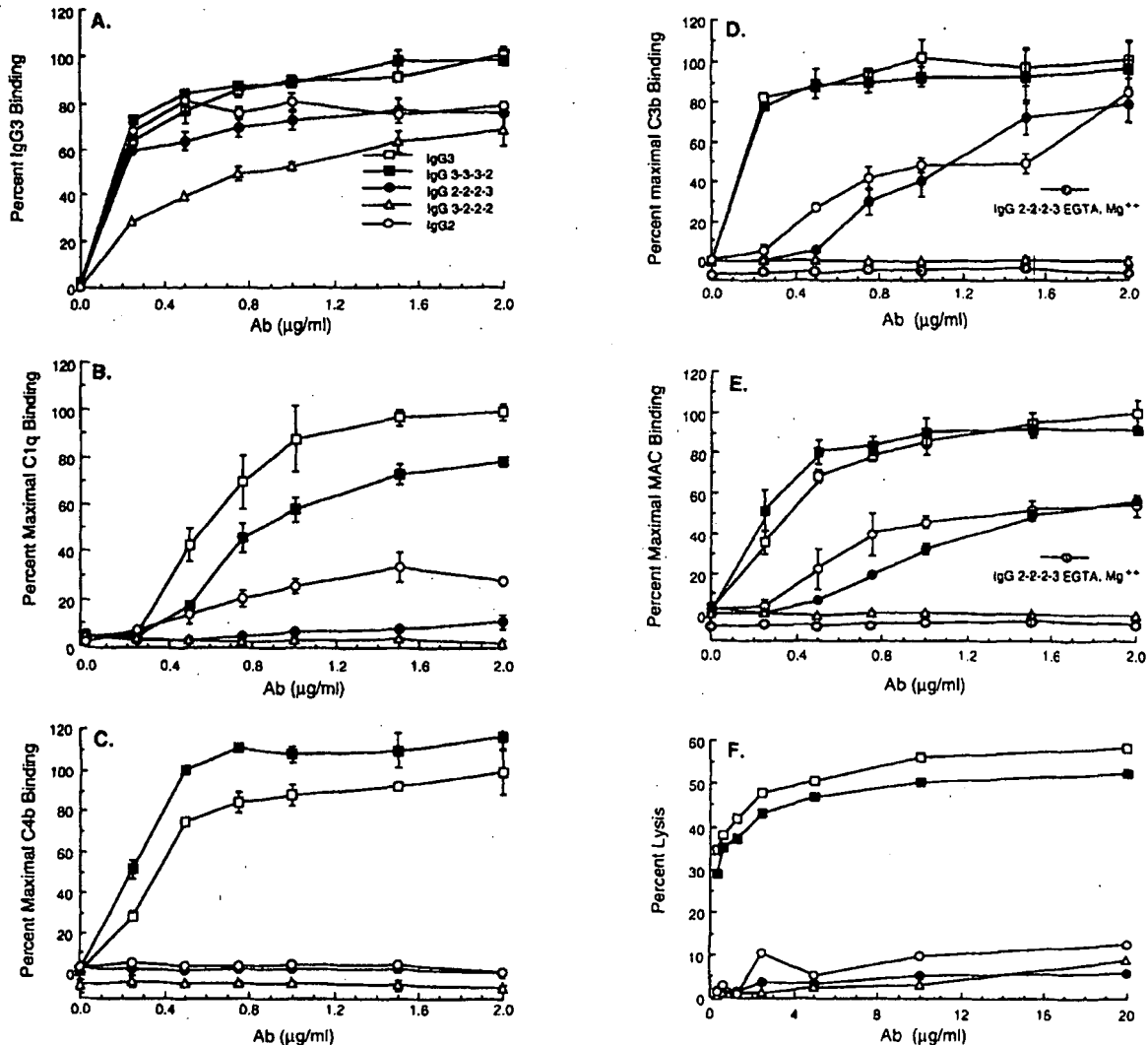
Fig. 9. Ag binding and complement activation by IgG2 mutants that contain $C_H1$, hinge or $C_H3$ from IgG3. For ELISAs, microtiter plates were coated with 0.5 μg/ml DNS-BSA. Antibodies (0 to 2 μg/ml) were incubated with Ag-coated plates overnight at 4°C. Human serum was added as the C' source. Bound C' components were detected with the appropriate goat anti-human C' component Ab, as described in Figs 3, 5, 6 and 7. For hemolysis, Ag-coated, ^{51}Cr-loaded SRBC were lysed with increasing concentrations of IgGs in the presence of human complement. Binding data were normalized to the maximum complement component binding induced by IgG3 and lysis data were calculated by comparison to total lysis of SRBC in water. (A) Ag binding; (B) C1q binding; (C) C4b binding; (D) C3b binding; (E) MAC binding; (F) complement-mediated hemolysis. For A–E, each data point represents the mean and standard deviation of triplicate measurements. For F, each data point represents the mean of duplicate measurements.

cleavage of C4 and C3 or alteration in the mutants of the optimal IgG binding sites for C4b and C3b. Although IgG2-2-2/3-2 and IgG2 were impaired in C1q binding and C1 activation, they were able to induce C4b and C3b binding. Perhaps the interaction with C1q is of low affinity, but nevertheless is adequate for triggering subsequent steps in the C' cascade. Indeed, we hypothesize that the variability we observed with proteins such as IgG2 and IgG 2-2-2/3-2 in some assays reflects low affinity interactions that occur at the limits of detection in our assays.

Consistent with this hypothesis, IgG2-2-2-3 also induced binding of C3b and the MAC in the absence of significant C1q and C4b binding and without activating the alternative pathway. Furthermore, the data indicate that IgG2 and IgG 2-2-2-3 are more effective at binding C1q and inducing activation and binding of C3 and MAC than the IgG2 mutant with the $C_H1$ of IgG3. Although the major C3 binding site for heat-aggregated IgG1 was shown to occur in the $C_H1$ (Shohet et al., 1993), it is possible that in the context of properly folded wild-type IgG2 or IgG3, this site is dependent on interactions with

other domains. Indeed, other investigators (Anton *et al.*, 1989) have proposed that both $C_H1$ and $C_H3$ may be involved in C3 binding. Interestingly, in the current study, the IgG2 mutant 3-2-2-2 failed to bind C1q, C4, C3 and the MAC, whereas the IgG3 mutant 3-3-3-2 was as effective as wildtype IgG3 in these activities. Together, data obtained with our mutant IgGs suggest that primary amino acid mutations may have induced changes in the tertiary IgG structure that affected either non-covalent or covalent interactions with C' components. Future studies with additional mutants should help to clarify these observations.

The observation that IgG2 and IgG2-2-2/3-2 do assemble MAC at reduced levels but do not mediate lysis may be relevant clinically. Sublytic MAC formation has been proposed to activate $Ca^{2+}$ signaling pathways in cells (Morgan, 1993) and was shown to induce degradation of myelin basic protein (Vanguri and Shin, 1988; Liu *et al.*, 1983). These effects may contribute to the pathogenesis of experimental allergic encephalomyelitis or multiple sclerosis. The ability of IgG2 and the mutants 2-2-2/3-2 and 2-2-2-3 to induce binding of C3 in the absence of directing lysis also may be important *in vivo*. For example, in diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and adult respiratory distress syndrome, production of fluid phase C3a and C5a, potent anaphylatoxins, by C'-activating immune complexes is thought to be a major contributor to inflammation (Kohl and Bitter-Suermann, 1993). At the same time, reduced binding of C3b may limit the ability of an IC containing IgG2 to be solubilized or cleared by CR1 and CR3. Precipitated ICs are thought to contribute to disease progression in SLE and RA (Ng and Schifferli, 1993). Thus, even though an IgG lacks the ability to activate the entire C' cascade, it may mediate other C'-associated effects. These findings underscore the importance of analysing multiple steps in the C' cascade when assessing Ab function.

The finding that the N-terminal residues of $C_H2$ are critical for C' activation is consistent with other findings. Recently, Morgan *et al.* (1995) showed that replacement of residues 233–237 in the N-terminus (lower hinge) of IgG1 with the sequence from IgG2 abolished the Ab's ability to direct C'-mediated lysis and to bind FcγRI and C1q. Also, C'-mediated lysis and C1q binding were abolished or reduced, respectively, by the single mutation of Leu 235 to Glu, implicating this specific residue, and suggesting that residues other than the C1q binding motif (Glu318, Lys320 and Lys322) identified by Duncan and Winter (1988) play a role in C1q binding and C' activation. Our data support these findings since all of the proteins examined contain the C1q biding motif, but optimal C1q binding is observed only with proteins containing the amino acids Glu233, Leu234, Leu235 and Gly236. Our data also suggest that domains other than $C_H2$ may play a role since some of our IgG2 domain-exchanged mutants were less active than wildtype IgG2.

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# Residue at Position 331 in the IgG1 and IgG4 $C_H2$ Domains Contributes to Their Differential Ability to Bind and Activate Complement*

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A conserved proline residue is found at position 331 in the $C_H2$ domains of human IgG subclasses which fix complement. This residue is replaced by a serine in IgG4 which is inactive. To determine the role of residue 331 in the differential ability of human IgGs to activate the complement cascade, a pair of genetically engineered anti-dinitrophenol IgG1 and IgG4 antibodies with reciprocal mutations at position 331 were tested for their hemolytic activity as well as for their ability to bind C1q, activate C1 and cleave C4. The IgG1 Ser[331] mutant was virtually unable to mediate the lysis of trinitrobenzenesulfonic acid-derivatized sheep red blood cells as a result of a marked defect in C1q binding activity. In contrast, the substitution of Pro for Ser[331] in IgG4 bestowed partial hemolytic activity (40%) to the IgG4 Pro[331] variant. Under low ionic strength conditions, this mutant was found to be approximately 50 and 75% as active as wild-type IgG1 in the C1q binding and C4b deposition assays, respectively. These results indicate that residue Pro[331], which folds into close proximity to a previously identified C1q binding motif (Duncan, A. R., and Winter, G. (1988) Nature 332, 738–740), contributes to the architecture of the IgG1 C1q binding site and that its replacement by a serine residue in IgG4 is largely responsible for the functional inactivity of this isotype.

In spite of their extensive amino acid sequence similarities, human IgG subclasses markedly differ in their abilities to activate the classical pathway of complement. IgG1 and IgG3 are the most efficient at binding and activating C1, IgG2 is significantly less active, and IgG4 is inactive (1). The observation that the Fcγ4 fragment binds C1 (2), whereas hinge-deleted proteins are unable to fix complement (3, 4), led to the hypothesis that, in molecules with restricted hinges, the Fab arms are brought in closer apposition to the Fc fragment and thus render the C1q binding site less accessible. It was initially believed that the segmental flexibility of IgG molecules depended on the length of their hinge region and correlated with complement activation (5, 6). However, later studies (7, 8) using genetically engineered IgG3 and IgG4 antibodies with either modified or switched hinge regions clearly demonstrated that the hinge was not responsible for the differences observed in the complement fixing ability of human IgG isotypes.

Early fragmentation studies revealed that the C1q binding site was located to the IgG1 $C_H2$ domain (9). This finding was unambiguously confirmed by experiments which showed that IgG1[1] and IgG3 (11) variants harboring an IgG4 $C_H2$ domain were no longer able to activate C1, whereas the grafting of either an IgG1 or IgG3 $C_H2$ domain onto IgG4 conferred C1 binding activity to the resulting hybrid molecule. In addition, the $C_H2$ carbohydrate moieties were found to be critical in maintaining the functional integrity of the C1 binding site, since aglycosylated IgGs do not fix complement (12, 13). A C1q binding motif (EKK), including 3 charged residues, Glu[318], Lys[320], and Lys[322] (Eu numbering) of the $C_H2$ domain, was identified by site-directed mutagenesis of a murine complement-fixing antibody (14). However, this motif is conserved among noncomplement-fixing antibodies. More recently, Tao et al. (11) engineered a set of IgG1/IgG4 hybrid antibodies in which the C-terminal halves of the IgG1 and IgG4 $C_H2$ domains were reciprocally exchanged and provided evidence that the C-terminal region of the $C_H2$ domain (residues 292–340) contains the residues responsible for the isotype-specific differences in complement activation. Interestingly, residues Ser[330] and Ser[331] within this region are IgG4-specific, whereas residues Ala[330] and Pro[331] are conserved among the other human IgG subclasses. Moreover, the analysis of the IgG1 Fc crystal structure (15) reveals that Pro[331] folds into proximity of the EKK C1q binding motif, and it has been shown that the mutation of its structural equivalent, residue Pro[436], in IgM (16) to Ser markedly decreased the binding affinity of IgM for C1q (17).

To determine the putative role of residue 331 in the IgG ability to bind and activate C1, we have engineered anti-DNP[2] chimeric IgG1 and IgG4 with reciprocal amino acid substitutions at this position. We have shown that a Pro[331] → Ser[331] substitution virtually abolished IgG1 ability to bind C1, whereas the reciprocal mutation bestowed significant, although partial, complement-fixing activity to IgG4.

## MATERIALS AND METHODS

*Cell Lines*—The murine myeloma λ chain-producing variant MOPC 315.26 cell line was maintained as described previously (18).

*Plasmid Constructions*—The 3.2-kilobase HindIII-BamHI Cγ1 and Cγ4 constant region genes were kindly provided by Dr. L. Hood (California Institute of Technology). The genes were cloned into the pEMBL19 vector. All molecular cloning techniques were performed according to Sambrook et al. (19). The reciprocal exchanges of residues

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[1] Y. Xu, M. Everett, S. Chappel, C. Horne, D. Isenman, K. J. Dorrington, and M. Klein, unpublished data.

[2] The abbreviations used are: DNP, dinitrophenol; GVB[2+], isotonic Veronal buffer containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$; PBS, phosphate-buffered saline; SRBCs, sheep red blood cells; TNBS, trinitrobenzenesulfonic acid; C, constant region: H, heavy chain; L, light chain; $V_H$, heavy chain variable region gene segment.

Pro[331](CCC → TCC) in IgG1 and Ser[331](TCC → CCG) in IgG4 (Eu numbering) were performed by site-directed mutagenesis of their respective codons using the method of Taylor *et al.* (20) (Amersham Corp.). The mutations were confirmed by DNA sequence analysis. The wild-type and mutated Cγ genes were then subcloned into the mammalian expression vector pSV2neo-V$_H$315, which contains the IgA MOPC 315 rearranged V$_H$ gene placed under the control of its own promoter (18). The resulting expression vectors encode an entire heavy chain containing the murine MOPC 315 V$_H$ domain and a human IgG constant region. All constructions were then introduced into MOPC 315.26 cells by electroporation (18). The transfected cells were grown and selected in α minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES, 100 µg of streptomycin/ml, and 100 units of penicillin/ml in the presence of 600 µg of G418/ml (Life Technologies, Inc.). The production of the chimeric IgG transfectomas was detected by an IgG-specific capture enzyme-linked immunosorbent assay, using purified human myeloma IgG1 and IgG4 as standards.

*Purification of Chimeric Anti-DNP IgG Proteins*—All recombinant IgG molecules were purified from culture supernatants by DNP-lysine affinity chromatography. After extensive dialysis against PBS, pH 7.2, the antibodies were further purified by gel chromatography on a Sephadex G-200 column. Purified antibodies (5 µg) were electrophoresed on a 10% SDS-polyacrylamide gel under both reducing and nonreducing conditions. The gel was stained with PAGE Blue 83 "Electran" (BDH, Toronto) and analyzed by scanning densitometry using an LKB Ultroscan XL densitometer. Antibody preparations were ultracentrifuged at 100,000 × *g* for 1 h to remove potential aggregates before all functional assays. Antibody concentrations were measured by the micro-BCA protein assay (Pierce Chemical Co.), using purified human myeloma IgG1 as a standard.

*Preparation of TNBS-SRBCs*—The preparation of TNBS-sensitized SRBCs was carried out as described by Rittenberg and Pratt (21). SRBCs (Woodlyn Labs, Guelph, Ontario, Canada) were washed three times with ice-cold PBS. Then, 20 mg of TNBS (Sigma) in 5 ml of PBS were added dropwise to a 1-ml suspension of 5 × 10⁹ SRBCs. The mixture was incubated for 20 min at room temperature with constant mixing. TNBS-coated cells were washed four times with ice-cold PBS and stored at a density of 1 × 10⁹ cells/ml at 4 °C in the dark.

*Complement-mediated Hemolysis*—TNBS-SRBCs (2 × 10⁷) were washed three times with isotonic GVB²⁺ (Veronal buffer containing 0.1% gelatin, 0.15 mM CaCl₂, and 0.5 mM MgCl₂) (µ = 0.15) and incubated with increasing amounts of purified IgGs (0.06–2 µg) for 30 min at 25 °C. GVB²⁺ alone was used as negative control. Unbound antibodies were removed by centrifuging the cells at 1,300 × *g* for 5 min at 4 °C and washing the cell pellets twice with 1 ml of ice-cold GVB²⁺. IgG-sensitized TNBS-SRBCs were then resuspended in 250 µl of GVB²⁺ and reacted with 100 µl of a 1/30 dilution of guinea pig complement (Diamedix, Miami, FL) preadsorbed with TNBS-SRBCs. Cell lysis was allowed to occur at 37 °C for 1 h. The reaction was terminated by adding 1 ml of ice-cold GVB²⁺ containing 10 mM EDTA. Unlysed TNBS-SRBCs were pelleted by centrifugation, and the degree of cell lysis was determined by measuring the optical density of the supernatant at 412 nm. All assays were performed in duplicate. Z values (Z = average number of hemolytic sites/cell) are expressed as the means of three independent determinations.

*¹²⁵I-C1q Binding Assay*—Human C1q was prepared by the method of Tenner et al. (22). Purified C1q was iodinated with carrier-free Na¹²⁵I (specific activity, 459.8 MBq/µg; Amersham Corp.) using the lactoperoxidase method as described by Wright *et al.* (17) to a specific activity of 710,000 cpm/µg. To prepare IgG-sensitized TNBS-SRBCs, 2 × 10⁷ TNBS-SRBCs were mixed with 2.5 µg of anti-DNP IgG in 200 µl of GVB²⁺ and kept on ice for 1 h with occasional shaking. Excess IgG was removed by washing the cells with ice-cold GVB²⁺ twice. Sensitized TNBS-SRBCs were then resuspended in 100 µl of low ionic strength SGVB²⁺ buffer containing 48.6% sucrose, 0.1% gelatin, 0.15 mM CaCl₂, and 0.5 mM MgCl₂ (µ = 0.06) and incubated with increasing amounts (0.01–1 µg) of ¹²⁵I-C1q in 100 µl of SGVB²⁺ at 30 °C for 30 min. The cell suspension was then layered onto a 200-µl dibutyl phthalate oil (Fisher) cushion in 400-µl microcentrifuge tubes. The tubes were centrifuged at 10,000 × *g* for 30 s, and the tips were cut. Cell-bound ¹²⁵I-C1q was measured by counting the cell pellet radioactivity in a γ counter. Nonspecific binding was determined using TNBS-SRBCs incubated with equivalent amounts of human myeloma IgG1. Each assay was performed in duplicate.

*¹²⁵I-C4b Deposition Assay*—Purified human C1 and C4 molecules were kindly donated by Dr. D. Isenman (University of Toronto). C4 was iodinated by the lactoperoxidase method to a specific activity of 840,000 cpm/µg. The ¹²⁵I-C4b deposition assay was performed according to the
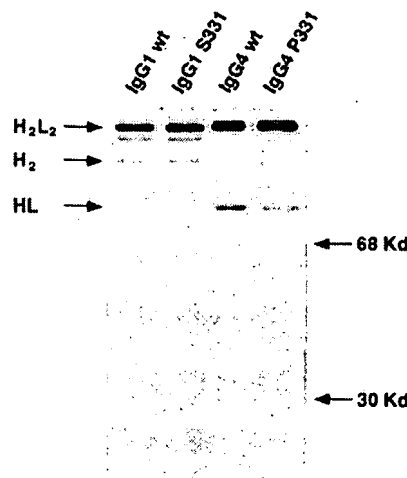


FIG. 1. **SDS-polyacrylamide gel analysis of purified wild-type and mutated anti-DNP antibodies.** Chimeric antibodies were purified by DNP affinity and Sephadex G-200 gel filtration chromatography from the supernatant of MOPC 315.26 transfectants. Purified samples (5 µg) were analyzed by 10% SDS-PAGE under nonreducing conditions. The electrophoretic mobilities of IgG tetramers and intermediate species are indicated.

protocol used for the ¹²⁵I-C1q binding assay, except that 100 µl of 3-fold dilutions (0.4–100 ng) of purified C1 in SGVB²⁺ were added to 100 µl of IgG-TNBS-SRBCs. After a 30-min incubation at 30 °C, the target cells were washed in SGVB²⁺ at 25 °C and reacted with a fixed amount of ¹²⁵I-C4 (800,000 cpm) in 200 µl of SGVB²⁺ at 30 °C for 30 min. Free ¹²⁵I-C4 was separated by centrifugation through an oil cushion, and the cell pellets were counted for radioactivity. Nonspecific binding was determined from the residual counts associated with TNBS-SRBCs incubated with equal amounts of human myeloma IgG1. Each assay was performed in duplicate. The amount of cell-bound ¹²⁵I-C4b was expressed as the mean of two independent experiments.

*Quantitation of Chimeric Antibodies Bound to TNBS-SRBCs*—Purified protein A (20 µg) (Pharmacia LKB Biotechnology Inc.) was labeled with Na¹²⁵I using the chloramine-T method to a specific activity of 1.5 × 10⁷ cpm/µg. Free Na¹²⁵I was removed by repeated centrifugations through Sephadex G-25 spin columns. To estimate the quantities of chimeric IgGs bound to TNBS-SRBCs, increasing amounts of chimeric IgGs (0.025–2 µg) were incubated with 2 × 10⁷ TNBS-SRBCs in 200 µl of GVB²⁺ as described for the complement-mediated lysis assay. IgG-TNBS-SRBCs were washed with 1 ml of ice-cold GVB²⁺, pelleted, and resuspended in 100 µl of GVB²⁺ containing a saturating amount of ¹²⁵I-protein A (250,000 cpm). After incubation for 30 min at 25 °C, cells were centrifuged through an oil cushion and pellets counted for radioactivity. Nonspecific binding was determined using TNBS-SRBCs mixed with an equal volume of GVB²⁺. All determinations were performed in duplicate.

*Molecular Modeling*—One C$_H$2 domain (Brookhaven Protein Data Bank entry 1fc1) (15) was used for energy minimization analysis of the Pro[331] → Ser[331] mutation, including residues 238–340 and the 9 crystallographically resolved carbohydrate residues of the *N*-linked sugar moieties. A Pro[331] → Ser[331] mutant was constructed by replacing the prolyl side chain with that of serine using the INSIGHT II software program (BIOSYM Technologies Inc., San Diego, CA). Both mutated and starting structures were minimized using conjugate gradients until the gradient of the system total energy was less than 0.01 Kcal.

### RESULTS

*Expression and Purification of Native and Mutated Chimeric IgG1 and IgG4 Antibodies*—High-producer transfectants secreting wild-type anti-DNP IgG1 and IgG4 as well as their reciprocal IgG1 Ser[331] and IgG4 Pro[331] mutants at levels between 1 and 16 µg/ml were maintained in G418-containing medium. SDS-PAGE analysis (Fig. 1) of the DNP affinity-purified antibodies revealed that all recombinant IgGs were >95% pure. IgG1s were essentially secreted as covalent H₂L₂ tetramers, although the trace amounts of H₂ and H₂L intermediates were detected. Wild-type IgG4 and the IgG4 Pro[331] mutant
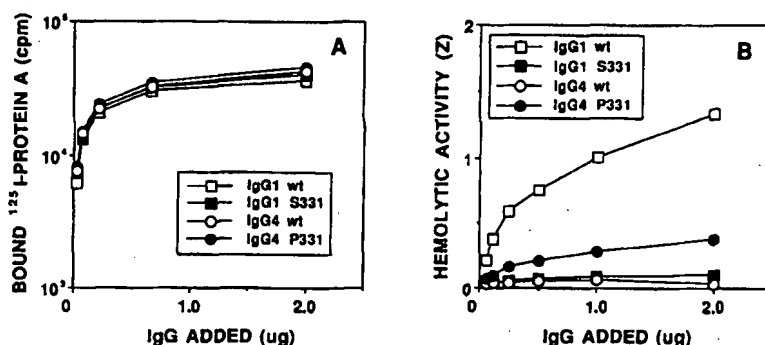
FIG. 2. **$^{125}$I-protein A binding and complement-mediated hemolysis of wild-type and mutated anti-DNP antibodies.** *A*, $^{125}$I-protein A (250,000 cpm) in GVB$^{2+}$ buffer were mixed with 2 × 10$^7$ IgG-TNBS-SRBCs prepared with increasing amounts (0.025–2 µg) of wild-type or mutated IgGs and incubated at room temperature for 30 min. Cell-bound and free ligands were separated by centrifugation through a cushion of dibutyl phthalate oil as described under "Materials and Methods." Each experimental point represents the mean of two determinations. *B*, 2 × 10$^7$ TNBS-SRBCs were lysed with increasing amounts (0.06–2 µg) of chimeric wild-type or mutated antibodies in the present of a 1/30 dilution of guinea pig complement (37 °C for 1 h). Each point represents the mean of two determinations.

consisted of a mixture of covalent tetramers (80–90%) and half-molecules (HL) (10–20%), as judged by scanning densitometry of the stained gel. All IgG species could be dissociated into their constitutive H and L chains upon reduction (data not shown).

*Complement-mediated Hemolysis*—To determine the role of residue 331 in complement binding and activation, reciprocal IgG1 (Pro$^{331}$ → Ser$^{331}$) and IgG4 (Ser$^{331}$ → Pro$^{331}$) mutants were engineered and assessed for their ability to lyse sensitized TNBS-SRBCs in the presence of an excess of guinea pig complement under physiological conditions. In preliminary experiments, TNBS-SRBCs were sensitized with increasing amounts of antibodies and reacted with an excess of $^{125}$I-protein A (Fig. 2*A*). Wild-type and mutated anti-DNP IgG1 and IgG4 antibodies were found to be equally efficient at sensitizing TNBS-SRBCs at all concentrations tested. The hemolytic activity of the various IgG species was then assessed in a dose-response complement activation assay. Wild-type chimeric IgG1 was found to be the most hemolytically active molecule whereas the IgG4 antibody was totally inactive (Fig. 2*B*). Interestingly, substitution of Pro$^{331}$ with Ser in the IgG1 variant virtually abolished its ability to activate the complement cascade (Fig. 2*B* and Table I), whereas the reciprocal replacement of Ser$^{331}$ with Pro in IgG4 conferred approximately 30% of wild-type IgG1 complement-fixing ability to the IgG4 Pro$^{331}$ variant.

*$^{125}$I-C4b Deposition Assay*—The ability of wild-type IgG1 and IgG4 and their respective variants to activate C1 and cleave C4 was then assessed in a C4b deposition assay. The cleavage of C4 into C4a and C4b by activated C1s results in the exposure of the C4b internal thioester bond. A small fraction of the large C4b fragment covalently binds to the antibody-sensitized SRBC surface or the Fab fragment of the antibody via trans-acylation onto hydroxyl and amino groups (23, 24). IgG-sensitized TNBS-SRBCs were preincubated with serial dilutions of purified human C1 and subsequently reacted with a fixed amount of $^{125}$I-C4. Since electrostatic interactions are involved in the C1q-IgG interaction (25), C1q binding is strongly dependent upon ionic strength (26, 27). Therefore, low ionic strength conditions (µ = 0.06) were used to facilitate C1 binding in the C4b deposition assay (17). As expected, $^{125}$I-C4b efficiently attached to IgG1-sensitized red cells in a dose-dependent manner (Fig. 3*A*). Approximately 10,000 C4b molecules per target cell were deposited in the presence of the highest amount (0.1 µg) of C1. No C4b deposition was observed with IgG4-sensitized targets. The amount of C4b bound to TNBS-derivatized red cells sensitized with the IgG1 Ser$^{331}$ mutant was markedly reduced from 10,000 to 2,700 (73%) molecules/cell as compared with wild-type IgG1 (Fig. 3*A* and Table I). In con-

trast, target erythrocytes coated with the IgG4 Pro$^{331}$ variant were found to be 75% as active (7,500 C4b molecules bound/cell) as IgG1-sensitized red cells at binding C4b.

*$^{125}$C1q Binding Assay*—A C1q binding assay was then performed to determine whether the marked decrease in $^{125}$I-C4b deposition observed with IgG1 Ser$^{331}$ was due to its inability to bind C1q or activate bound C1. Fig. 3*B* shows that C1q efficiently bound to wild-type IgG1 in a dose-dependent manner. In the presence of 1 µg of $^{125}$I-C1q, one IgG1-TNBS-SRBC bound 6,400 C1q molecules (Table I). No binding was detected with IgG4, in spite of the low ionic strength conditions used in the assay. A significant decrease in $^{125}$I-C1q binding (3-fold) was observed with IgG1 Ser$^{331}$, whereas C1q binding was restored up to approximately 50% in IgG4 Pro$^{331}$ as compared with IgG1 (Fig. 3*B* and Table I).

*Molecular Modeling Studies*—No significant conformational differences could be detected in the hinge-proximal C$_H$2 loops of the minimized IgG1 and IgG1 Ser$^{331}$ structures. In each case, the loop moves out and away from the body of the C$_H$2 domain, although it is bent slightly more toward the rest of the domain in the mutant. This may be due to increased hydrogen bonding capabilities of serine at position 331. The hydroxyl group of Ser$^{331}$ is a possible hydrogen bond donor to the carbonyl group of Pro$^{329}$ and acceptor for the side chain of Ser$^{324}$. The conformation of the C1q binding motif residues Glu$^{318}$, Lys$^{320}$, and Lys$^{322}$ (Fig. 4) was essentially unchanged in both wild-type IgG1 and the IgG1 Ser$^{331}$ variant.

## DISCUSSION

Human IgG isotypes markedly differ by their ability to activate the classical pathway of complement. Residues (292–340) localized within the C-terminal region of the C$_H$2 domain were found to be responsible for the isotype-specific differences in complement activation (11). Two residues, Ala$^{330}$ and Pro$^{331}$, within this region are highly conserved among human IgG subclasses with complement-fixing ability. These residues are replaced by Ser$^{330}$ and Ser$^{331}$, respectively, in the inactive IgG4 molecule. Wright *et al.* (17) reported that the C1q binding site of IgM was localized within its Cµ3 domain and that the replacement of residue Pro$^{436}$ (Ou index), which is the structural equivalent of Pro$^{331}$ in IgG1 (16), by Ser$^{436}$ markedly impaired the ability of the pentamer to bind and activate C1. We therefore engineered a pair of anti-DNP IgG1 and IgG4 molecules with reciprocal point mutations at position 331 to determine the role of this residue in modulating the complement-fixing ability of human IgG subclasses.

Wild-type anti-DNP IgG1 was, as expected, the most efficient

TABLE I

*Relative hemolytic, C1q, and C4b binding activities of wild-type and mutated chimeric IgG antibodies*

| IgG species | Hemolytic activity (Z)[a] | Bound C1q[b] | | C4b deposition[c] | |
|---|---|---|---|---|---|
| | | ng | Molecules/cell | ng | Molecules/cell |
| IgG1 wt | 1.000 | 98 ± 4.2 | 6,400 ± 274 (100%) | 67 ± 3.3 | 10,000 ± 493 (100%) |
| IgG1 Ser[331] | 0.009 | 29 ± 1.3 | 1,900 ± 85 (29%) | 18 ± 0.9 | 2,700 ± 135 (27%) |
| IgG4 wt | 0.006 | UD[d] | UD | UD | UD |
| IgG4 Pro[331] | 0.281 | 48 ± 5.3 | 3,100 ± 342 (48%) | 50 ± 2.2 | 7,500 ± 330 (75%) |

[a] Z values are calculated for 1 µg of IgG and expressed relative to that of wild-type IgG1 taken as 1.000.

[b] Amounts of cell-bound C1q per 2 × 10^{7} TNBS-SRBCs sensitized with 2.5 µg of IgG antibodies and reacted with 1 µg of purified C1q. Results are expressed as the means of two determinations ± S.E.

[c] Amounts of cell-bound C4b per 2 × 10^{7} TNBS-SRBCs sensitized with 2.5 µg of IgG antibodies and reacted with 0.1 µg of purified C1. Assays were performed in duplicate and results are expressed as the means of two independent determinations ± S.E.

[d] UD, undetected.
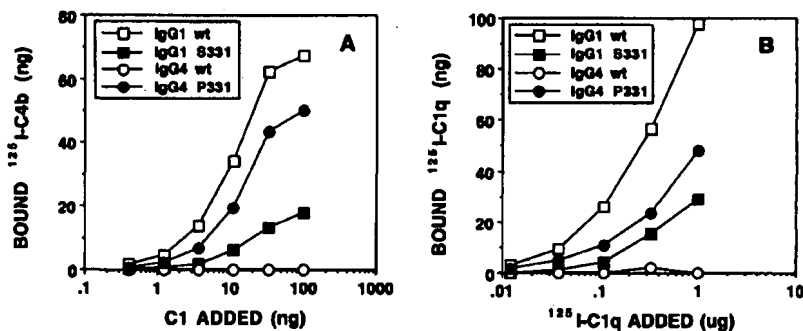


Fig. 3. **Deposition of ^{125}I-C4b on and binding of ^{125}I-C1q to IgG-TNBS-SRBCs.** TNBS-SRBCs (2 × 10^{7}) in GVB[2+] (µ = 0.15) were preincubated with 2.5 µg of either wild-type or mutated chimeric IgG antibodies. *A*, TNBS-SRBCs were then mixed with serial dilutions (0.4–100.0 ng) of purified human C1 (30 °C, 30 min) and subsequently reacted with a fixed amount of ^{125}I-C4 in SGVB[2+] (µ = 0.06) at 30 °C for 30 min. The amount of target-bound [125]C4b was calculated as described under "Materials and Methods." Each assay was performed in duplicate, and each point represents the mean of two independent determinations. *B*, sensitized erythrocytes were reacted with increasing amounts of ^{125}I-C1q (0.01–1 µg) in SGVB[2+] at 30 °C for 30 min. The amount of bound C1q was determined as described under "Materials and Methods." Each point represents the mean of two determinations.

FIG. 4. *A*, ribbon diagram of the Fc domain from Brookhaven Protein Data Bank entry 1fc1 with *N*-linked oligosaccharide separating the two C_H2 domains. The lower hinge region (residues 229–237) is a model (R. Oomen, unpublished results) to indicate the approximate location of Leu[234] relative to residue His[268], Glu[318], Lys[320], Lys[322], Ala[330], and Pro[331]. Van der Waals surfaces are shown for Tyr[296] on the opposite domain. *B*, all-atom (except hydrogens) detail of the *boxed* C_H2 domain from *A*, in the same orientation. The carbohydrate moieties and labeled side chains are drawn with *heavy lines*. A solvent-accessible surface was calculated for the labeled residues using a 1.4 Å probe according to Connolly (10). The solvent-accessible surface of residues Ala[330], Pro[331], and those forming the EKK C1q binding motif are clearly contiguous.
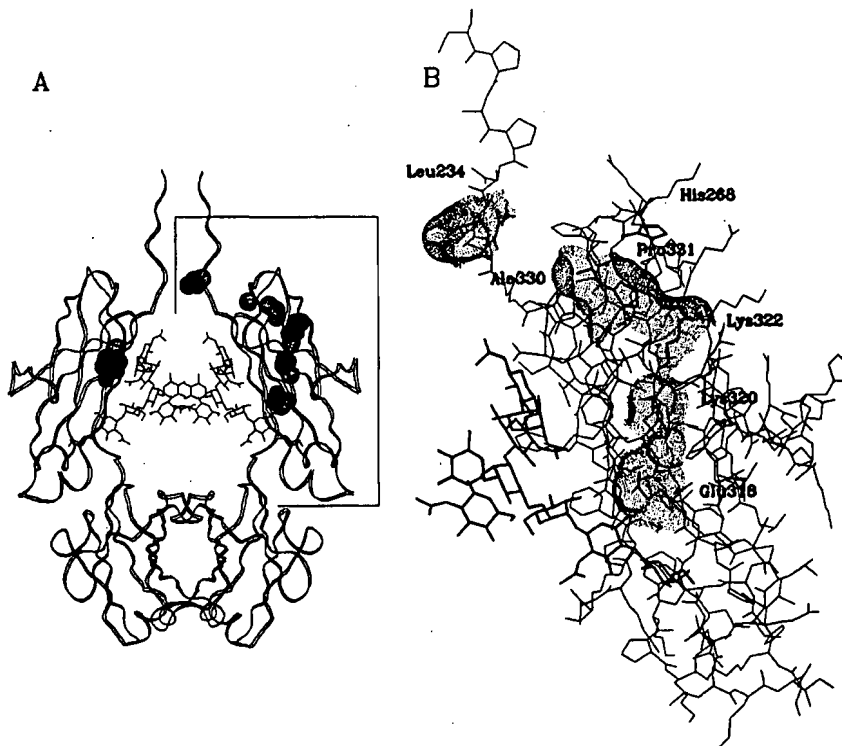


antibody at lysing TNBS-SRBCs, whereas chimeric wild-type IgG4 was totally inactive. Since the degree of C1 activation and the extent of C4 cleavage depend upon the antibody isotype and do not necessarily correlate (28, 29), both the C1q binding and

C4b deposition assays were performed in parallel to determine the mechanisms responsible for the changes in hemolytic activities observed with the IgG1 Ser[331] and IgG4 Pro[331] mutants. The Pro[33] → Ser[331] substitution in IgG1 completely abol-

ished its hemolytic activity. Under low ionic strength conditions, the defective IgG1 Ser[331] mutant exhibited a marked decrease (73%) in its ability to cleave C4 as a result of a proportional reduction (71%) in C1q binding activity. In contrast, the substitution of Ser[331] with Pro conferred 50% C1q binding activity to IgG4 Pro[331] relative to wild-type IgG1 and an even more significant ability (75%) to fix C4b. This is reminiscent of the findings by Bindon *et al.* (28, 29) that, at comparable levels of C1 binding, some IgG isotypes either activate C4 faster or are better substrates for the covalent attachment of C4b. Taking into account the fact that reduced IgGs do not bind C1 (2) and that IgG4 Pro[331] contained approximately 15% of half-molecules, its actual hemolytic activity was calculated to be 40% that of native IgG1.

These results clearly indicate that Pro[331] plays an important role in conferring complement-fixing ability to IgG isotypes. This residue of the hinge-proximal C$_H$2 loop folds adjacent to residue Lys[322] which is part of the EKK C1q binding motif (15) (Fig. 4B). Molecular modeling supports the premise that the Pro[331] → Ser[331] substitution did not dramatically alter the secondary structure of this loop, but suggests that serine may introduce local interactions such as hydrogen bonding, which may preclude contact with a complementary surface on C1q. Although residues Glu[318], Lys[320], and Lys[322] are likely involved in the formation of electrostatic bonds between C1q and IgG1 (25, 30), their functional role in C1q binding must be modulated by other residues, since they are conserved among both complement-fixing and complement nonfixing antibodies. The functional role of Pro[331] could then be to facilitate C1q docking. However, substitution of Ser[331] with Pro could not confer a full IgG1-like hemolytic activity to IgG4 Pro[331]. Hinge exchange experiments revealed that the grafting of the short IgG4 hinge region onto IgG3 restricted its segmental flexibility (7) but did not alter the ability of either hinge-modified IgG3 (7, 8, 31) or IgG1[1] antibodies to activate complement. Therefore, other IgG1-specific residues within the C$_H$2 domain must contribute to the architecture of the C1q binding site. We have shown that replacement of Leu[234] in the IgG1 "hinge link" with the IgG4-specific residue Phe[234] dramatically reduced IgG1 complement-fixing activity; however, the reciprocal substitution did not confer C1 binding to the IgG4 Leu[234] mutant.[1]

Besides Pro[331], other residues on the surface of the C$_H$2 domain are thought to contribute to C1q binding. One such residue is the neighboring IgG1 amino acid Ala[330], which is replaced with a serine in IgG4. Ala[330], which is solvent-exposed, could either interact directly with C1q or play a role at presenting the side chain of residue 331 in the proper orientation. The greater ($\phi$, $\Psi$) space of alanine, as compared with residues with longer side chains, allows the main chain to adopt conformations that can accommodate subsequent sterically restricting proline residues. Residues 330 and 331 extend the contiguous patch of the solvent-exposed surface of the EKK motif to about 280 Å (Fig. 4). Flexibility in the lower hinge could also bring the Leu[234] side chain into proximity with the Pro[331]-containing loop, thereby extending the surface even more. However, this hypothesis cannot account for the fact that IgG2, which contains both the Ala[330]-Pro[331] dipeptide and the EKK motif, has an extremely low affinity for C1q (28, 32).

The tyrosine found at position 296 in IgG1 and IgG3 subclasses is replaced by phenylalanine in the complement nonfixing IgG2 and IgG4 isotypes. It has thus been proposed (11) that Tyr[296], which is located on the X face of the C$_H$2 domain opposite to the C1q binding site (Fig. 4A), might contribute to its architecture, since both C$_H$2 domains were believed to be required for C1 activation (33). However, more recent studies with hybrid IgGs suggest that only one H chain is necessary for C1 binding (34).

Thus, residues 234, 331, and perhaps 330 contribute to the C1q binding site and are, at least in part, responsible for the isotype-specific differences in complement activation. Together with the EKK binding motif they form a solvent-exposed patch likely recognized by the globular C1q heads. This contiguous patch may be partially hindered in hinge-deleted IgG1 and IgG3 molecules which lack inter-heavy chain disulfide bridges (3, 4) by the closer apposition of the Fab arms relative to Fc region. However, the recent finding that a hinge-deleted IgG3 with engineered disulfide bonds bridging its heavy chains can fix complement (35) confirms the early hypothesis (2) that a covalent linkage between immunoglobulin heavy chains is required for complement-fixing activity. Previous studies have clearly demonstrated that complement fixation was abolished or 50% reduced in aglycosylated immunoglobulins (12, 13) and IgGs lacking C$_H$3 (33, 36), respectively. In this regard, structural perturbations in the vicinity of the His[268] (Fig.4B) reporter group were detected by [1]H NMR in aglycosylated IgG3 molecules (37, 38), indicating that the removal of the N-linked oligosaccharides induces a certain degree of distortion in the C$_H$2 hinge-proximal loop. Taken together, these results suggest that both the clipping of the C$_H$2 domains by the hinge disulfides and the paired C$_H$3 domains and the presence of carbohydrates are necessary to stabilize their native orientation and to preserve the functional integrity of the C1q binding site.

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L4       39281 L3 AND CHIMERIC


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L5        1869 L4 AND MODIFIED


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L7   ANSWER 1 OF 7  CAPLUS  COPYRIGHT 2007 ACS on STN
2007:84682   Document No. 146:182963  Preparation and expression of genetically
     modified human IgG1 and IgG3 antibodies in host cells
     having defective GDP-fucose synthesis-associated enzyme.  Shitara, Kenya;
     Niwa, Rinpei; Natsume, Akito (Kyowa Hakko Kogyo Co., Ltd., Japan).  PCT
     Int. Appl. WO 2007011041 A1 20070125, 162pp.  DESIGNATED STATES: W:  AE,
     AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR,
     CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR,
     HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS,
     LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM,
     PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN,
     TR, TT, TZ, UA, UG, US, UZ, VC; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM,
     CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IS, IT, LU, MC, ML, MR, NE, NL,
     PT, SE, SN, TD, TG, TR.  (Japanese).  CODEN: PIXXD2.  APPLICATION: WO
     2006-JP314531 20060721.  PRIORITY: JP 2005-212979 20050722; JP 2006-108216
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20060411.
AB  A human IgG1 **antibody** having the substitution of a polypeptide
    including a **CH2 domain** present in the Fc region by a
    polypeptide comprising an amino acid sequence corresponding to the amino
    acid sequence present at the same position as numbered according to the EU
    index as in Kabat et al. in a human IgG3 **antibody**; a genetically
    **modified antibody** composition having a higher
    complement-dependent cytotoxic activity than a human IgG3 **antibody**
    ; an **antibody** mol. contained in the genetically **modified**
    **antibody** composition; DNA encoding the heavy-chain constant region of the
    **antibody** mol.; a transformant produced by introducing the DNA into
    a host cell; a method for production of a genetically **modified**
    **antibody** composition using the transformant; and a pharmaceutical
    comprising the genetically **modified antibody** composition as
    an active ingredient.  Thus recombinant human IgG1 or IgG3
    **antibodies** were prepared for diagnostic and therapeutic uses and
    were expressed in host cells having lowered or inactivated GDP-fucose
    synthesis-associated enzyme such as GDP-mannose-4,6-dehydratase,
    GSP-4-keto-6-deoxy-D-mannose-3,5-epimerase or $\alpha$-1,6-
    fucosyltransferases.

L7  ANSWER 2 OF 7      MEDLINE on STN                    DUPLICATE 1
2006576201.    PubMed ID: 17006638.    Galactosylation of IgG from rheumatoid
    arthritis (RA) patients--changes during therapy. Pasek Marta; Duk Maria;
    Podbielska Maria; Sokolik Renata; Szechinski Jacek; Lisowska Elwira;
    Krotkiewski Hubert. (Ludwik Hirszfeld Institute of Immunology &
    Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114,
    Wroclaw, Poland. ) Glycoconjugate journal, (2006 Nov) Vol. 23, No. 7-8,
    pp. 463-71.  Journal code: 8603310. ISSN: 0282-0080. Pub. country: United
    States. Language: English.
AB  It is well documented that serum IgG from rheumatoid arthritis (RA)
    patients exhibits decreased galactosylation of its conservative N-glycans
    (Asn-297) in **CH2 domains** of the heavy chains; it has
    been shown that this agalactosylation is proportional to disease severity.
    In the present investigation we analyzed galactosylation of IgG derived
    from the patients using a **modified** ELISA-plate test, biosensor
    BIAcore and total sugar analysis (GC-MS).  For ELISA and BIAcore the
    binding of IgG preparations, purified from the patients' sera, to two
    lectins: Ricinus communis (RCA-I) and Griffonia simplicifolia (GSL-II) was
    applied.  Based on ELISA-plate test an agalactosylation factor (AF, a
    relative ratio of GSL-II/RCA-I binding) was calculated, which was
    proportional to actual disease severity.  Repeated testing of several
    patients before and after treatment with methotrexate (MTX) alone or in
    combination with Remicade (a **chimeric antibody**
    anti-TNF-alpha) supplied results indicating an increase of IgG
    galactosylation during the treatment.  This introductory observation
    suggests that IgG galactosylation may be an additional indicator of the RA
    patients' improvement.

L7  ANSWER 3 OF 7  CAPLUS  COPYRIGHT 2007 ACS on STN
2005:612335   Document No. 143:139052  Sequences for highly sialylated
    Fc-erythropoietin fusion protein with improved pharmacokinetics.  Gillies,
    Stephen D.; Lauder, Scott (Merck Patent G.m.b.H., Germany).  PCT Int.
    Appl. WO 2005063808 A1 20050714, 87 pp.  DESIGNATED STATES: W:  AE, AG,
    AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU,
    CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID,
    IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
    MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,
    SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN,
    YU, ZA, ZM, ZW; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES,
    FI, FR, GA, GB, GR, IE, IS, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD,
    TG, TR.  (English).  CODEN: PIXXD2.  APPLICATION: WO 2004-EP14608
    20041222.  PRIORITY: US 2003-533858P 20031231.
AB  The present invention provides novel highly sialylated Fc-erythropoietin
    (EPO) fusion proteins preferably comprising a couple of modifications in

the Fc- portion as well as in the EPO portion and having improved
pharmacokinetics. Specifically, the Fc-EPO proteins have a prolonged
serum half-life and increased in vivo potency. The Fc-EPO fusion proteins
synthesized in BHK cells have dramatically prolonged serum half-lives and
increased in vivo potency when compared to corresponding Fc-EPO fusion
proteins produced in other cell lines, such as, for example, NS/O cells.
The present invention provides Fc-EPO proteins synthesized in BHK, NS/0,
PerC6, or 293 cells.

L7    ANSWER 4 OF 7  CAPLUS  COPYRIGHT 2007 ACS on STN
2004:412975   Document No. 140:422398  Adjuvant acty of carrier proteins
    conjugated to **antibodies** against CD40 or CD28. Heath, Andrew
    (Adjuvantix Limited, UK). PCT Int. Appl. WO 2004041866 A1 20040521, 51
    pp. DESIGNATED STATES: W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR,
    BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
    GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
    LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM,
    PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT,
    TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW; RW: AT, BE, BF, BJ, CF, CG,
    CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR,
    NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION:
    WO 2003-GB4738 20031103. PRIORITY: GB 2002-25736 20021105.
AB    The author discloses an adjuvant comprising a conjugate of carrier and an
    **antibody** directed against CD28 or CD40. The adjuvant is used in a
    vaccine composition to immunize animals, typically but not exclusively, against
    T-cell independent antigens; the T-cell independent antigen itself
    comprising a conjugate with the above carrier. In one example, the
    carrier is tetanus toxoid.

L7    ANSWER 5 OF 7  CAPLUS  COPYRIGHT 2007 ACS on STN
1999:691228   Document No. 131:321542  **Chimeric** immunoglobulins
    contg. CH domains of IgA. Morrison, Sherie L.; Chintalacharuvu, Koteswara
    R.; Yoo, Esther Mikyung; Trinh, Kham M.; Coloma, M. Josefina (The Regents
    of the University of California, USA). PCT Int. Appl. WO 9954484 A1
    19991028, 67 pp. DESIGNATED STATES: W:  AE, AL, AM, AT, AU, AZ, BA, BB,
    BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM,
    HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,
    LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,
    SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD,
    RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI,
    FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG.
    (English). CODEN: PIXXD2. APPLICATION: WO 1999-US8647 19990420.
    PRIORITY: US 1998-82578 19980420; US 1998-96085 19980811.
AB    The authors disclose the preparation of **modified** Ig mols. using exon
    exchange. In one example, the **modified antibody** in
    IgG2 and contains a CH3 domain of an IgA mol. (α CH3). The
    combination of an α CH3 with other domains selected from one or more
    non-IgA **antibodies** provides for an Ig mol. that has the capacity
    to bind J chain and/or secretory component together with features of a
    non-IgA **antibody**. The **modified** Igs can also contain a
    CH1 and/or a **CH2 domain** of an IgA mol. The
    combination of an α CH1 and/or a **CH2 domain** with
    other domains selected from one or more non-IgA **antibodies**
    provides for the capacity to form higher polymers (trimers, tetramers,
    pentamers, etc.). The **chimeric antibodies** can also be
    engineered to lack one or more carbohydrate addition sites.

L7    ANSWER 6 OF 7     MEDLINE on STN                    DUPLICATE 2
92232133.   PubMed ID: 1567557.  Mapping rheumatoid factor binding sites
    using genetically engineered, **chimeric IgG antibodies.**
    Bonagura V R; Artandi S E; Agostino N; Tao M H; Morrison S L. (Department
    of Microbiology, College of Physicians and Surgeons, Columbia University,
    New York, NY 10032. ) DNA and cell biology, (1992 Apr) Vol. 11, No. 3, pp.
    245-52. Journal code: 9004522. ISSN: 1044-5498. Pub. country: United
    States. Language: English.

AB   We are using **chimeric** IgG **antibodies** consisting of
     murine variable regions joined to human constant regions as rheumatoid
     factor (RF) binding substrates to localize and map IgM RF binding sites on
     IgG.  Using **chimeric antibodies** in a **modified**
     RF ELISA, we showed that RFs from rheumatoid arthritis (RA) and
     Waldenstrom's macroglobulinemia (WMac) patients differ in their binding
     specificities for IgG3, although some of these RFs share common
     specificity for IgG1, IgG2, and IgG4.  By shuffling constant region
     domains between IgG3 and IgG4, we showed that sequence variation in the
     CH3 domain is responsible for WMac-derived RF differentiation of IgG3 and
     IgG4.  By making site-directed mutations in the wild-type IgG3 or IgG4
     human gamma constant genes, we showed that His-435 is an essential residue
     in RF binding to IgG for most WMac RFs.  The allotypic polymorphism in
     IgG3 at 436 is not responsible for differences in previous reports of
     high-frequency IgG3 binding by WMac RFs.  A amino acid loop in the
     **CH2 domain** of IgG4 proximal to the CH2-CH3 interface is
     important in WMac RF binding to IgG; a more distal CH2 loop in CH2 has a
     more variable effect on WMac RF binding.  To evaluate the contribution of
     the N-linked carbohydrate moiety at Asn-297 to RF binding sites on IgG, we
     measured RF binding to aglycosylated IgG **antibodies** produced by
     mutating the glycosylation signal Asn-297 to another amino acid.  Of all
     four IgG subclasses, only aglycosylated IgG3 was a better RF binding
     substrate than its glycosylated subclass counterpart.(ABSTRACT TRUNCATED
     AT 250 WORDS)

L7   ANSWER 7 OF 7  CAPLUS  COPYRIGHT 2007 ACS on STN
1990:476476   Document No. 113:76476  **Antibodies** having
     **modified** carbohydrate content and methods of preparation and use.
     Morrison, Sherie L.; Oi, Vernon T.; Hinton, Paul R. (Columbia University,
     USA; Becton, Dickinson and Co.).  Eur. Pat. Appl. EP 359096 A1 19900321,
     23 pp.  DESIGNATED STATES: R:  AT, BE, CH, DE, ES, FR, GB, GR, IT, LI, LU,
     NL, SE.  (English).  CODEN: EPXXDW.  APPLICATION: EP 1989-116368 19890905.
     PRIORITY: US 1988-244744 19880915.
AB   A method of altering the affinity of an **antibody** for the antigen
     to which it is directed comprises introducing into the variable region of
     the **antibody** a carbohydrate recognition site under conditions
     such that a carbohydrate binds to the site.  The carbohydrate content is
     also **modified** by deleting from a constant region of the
     **antibody** a carbohydrate recognition site which naturally occurs in
     the constant region.  The **antibodies** can be labeled, attached to a
     solid support, or conjugated with therapeutic ligands for use in anal.,
     affinity chromatog., and therapy.  The carbohydrate site in the
     **CH2 domain** of human IgG subclasses was deleted by
     site-directed mutagenesis of the DNA encoding the IgGs.  The resultant
     **antibodies** had decreased ability to bind Fc receptors and to
     activate complement.


=> s 13 and reduced complement lysis
L8            0 L3 AND REDUCED COMPLEMENT LYSIS

=> s 13 and effector function
L9        6719 L3 AND EFFECTOR FUNCTION

=> s 19 and CH2 domain
L10         87 L9 AND CH2 DOMAIN

=> s 110 complement
MISSING OPERATOR L10 COMPLEMENT
The search profile that was entered contains terms or
nested terms that are not separated by a logical operator.

=> s 110 and complement
L11         44 L10 AND COMPLEMENT

```
=> dup remove l11
PROCESSING COMPLETED FOR L11
L12            28 DUP REMOVE L11 (16 DUPLICATES REMOVED)

=> s l12 and substitution
L13            4 L12 AND SUBSTITUTION

=> dup remove l13
PROCESSING COMPLETED FOR L13
L14            4 DUP REMOVE L13 (0 DUPLICATES REMOVED)

=> d l14 1-4 cbib abs
```

L14   ANSWER 1 OF 4      MEDLINE on STN
95309977.     PubMed ID: 7540592.    Activation of **effector**
      **functions** by immune complexes of mouse IgG2a with isotype-specific
      autoantibodies. Rajnavolgyi E; Fazekas G; Lund J; Daeron M; Teillaud J L;
      Jefferis R; Fridman W H; Gergely J. (Department of Immunology, L. Eotvos
      University, God, Hungary. ) Immunology, (1995 Apr) Vol. 84, No. 4, pp.
      645-52. Journal code: 0374672. ISSN: 0019-2805. Pub. country: ENGLAND:
      United Kingdom. Language: English.
AB    Analysis of five monoclonal autoantibodies, rheumatoid factors produced by
      hybridomas generated from spleen cells of BALB/c mice repeatedly infected
      with A/PR/8/34 human influenza A virus, revealed that they recognized
      distinct but spatially related epitopes.  The differing isoallotypic
      specificity of the IgM and IgA monoclonal **antibodies** correlated
      with the presence of Ile258 and Ala305, respectively.  Although these data
      suggest that the epitopes recognized are within the **CH2**
      **domain**, all **antibodies** failed to inhibit IgG antigen
      reactivity with Staphylococcus aureus protein A (SpA), C1q, mouse C3,
      human Fc gamma RI or mouse Fc gamma RII, activities known to be
      predominantly determined by **CH2 domain** structures.
      Reactivity of the IgA **antibody**, Z34, with IgG2b allowed further
      specificity studies using a panel of 26 mutant IgG2b proteins, each having
      single amino acid replacements over the surface of the **CH2**
      **domain**.  The only **substitution** that affected Z34
      reactivity was Asn/Ala297, which destroyed the glycosylation sequon,
      resulting in secretion of an aglycosylated IgG molecule.  The epitope
      recognized by Z34 therefore seems to be located outside of the Fc gamma R
      and C1q binding sites, but to be dependent on the presence of carbohydrate
      for expression.  In contrast to the binding studies, **complement**
      activation by aggregated IgG2a, through classical or alternative pathways,
      was inhibited by the presence of autoantibodies.  The functional
      significance of isotype-specific autoantibody in immune regulation is
      discussed.

L14   ANSWER 2 OF 4   SCISEARCH   COPYRIGHT (c) 2007 The Thomson Corporation   on
      STN
1994:70790    The Genuine Article (R) Number: MV631. RESIDUE AT POSITION-331 IN
      THE IGG1 AND IGG4 **CH2 DOMAINS** CONTRIBUTES TO THEIR
      DIFFERENTIAL ABILITY TO BIND AND ACTIVATE **COMPLEMENT**.   XU Y
      (Reprint); OOMEN R; KLEIN M H. UNIV TORONTO, DEPT IMMUNOL, MED SCI BLDG,
      TORONTO M5S 1A8, ONTARIO, CANADA; CONNAUGHT CTR BIOTECHNOL RES, N YORK M2R
      3T4, ON, CANADA; UNIV TORONTO, DEPT BIOCHEM, TORONTO M5S 1A8, ONTARIO,
      CANADA. JOURNAL OF BIOLOGICAL CHEMISTRY (4 FEB 1994) Vol. 269, No. 5, pp.
      3469-3474. ISSN: 0021-9258. Publisher: AMER SOC BIOCHEMISTRY MOLECULAR
      BIOLOGY INC, 9650 ROCKVILLE PIKE, BETHESDA, MD 20814. Language: English.
      *ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS*
AB       A conserved proline residue is found at position 331 in the C(H)2
      domains of human IgG subclasses which fix **complement**.  This
      residue is replaced by a serine in IgG4 which is inactive.  To determine
      the role of residue 331 in the differential ability of human IgGs to
      activate the **complement** cascade, a pair of genetically
      engineered anti-dinitrophenol IgG1 and IgG4 **antibodies** with
      reciprocal mutations at position 331 were tested for their hemolytic

activity as well as for their ability to bind C1q, activate C1 and cleave
C4.  The IgG1 Ser331 mutant was virtually unable to mediate the lysis of
trinitrobenzene-sulfonic acid-derivatized sheep red blood cells as a
result of a marked defect in C1q binding activity.  In contrast, the
**substitution** of Pro for Ser331 in IgG4 bestowed partial hemolytic
activity (40%) to the IgG4 Pro331 variant.  Under low ionic strength
conditions, this mutant was found to be approximately 50 and 75% as active
as wild-type IgG1 in the C1q binding and C4b deposition assays,
respectively.  These results indicate that residue Pro''', which folds
into close proximity to a previously identified C1q binding motif (Duncan,
A.  R., and Winter, G. (1988) Nature 332, 738-740), contributes to the
architecture of the IgG1 C1q binding site and that its replacement by a
serine residue in IgG4 is largely responsible for the functional
inactivity of this isotype.

L14  ANSWER 3 OF 4   CAPLUS  COPYRIGHT 2007 ACS on STN.
1989:207251   Document No. 110:207251  Altered **antibodies** having
      altered **effector functions** and their preparation.
      Winter, Gregory Paul; Duncan, Alexander Robert; Burton, Dennis Raymond
      (Medical Research Council, UK).  PCT Int. Appl. WO 8807089 A1 19880922, 42
      pp.  DESIGNATED STATES: W:  AU, GB, JP, US; RW: AT, BE, CH, DE, FR, GB,
      IT, LU, NL, SE.  (English).  CODEN: PIXXD2.  APPLICATION: WO 1988-GB211
      19880318.  PRIORITY: GB 1987-6425 19870318; GB 1987-18897 19870810; GB
      1987-28042 19871201.
AB    **Antibodies** (Ab) with altered binding affinity for effectors such
      as the C1q component of the **complement** system are prepared by
      replacing amino acid residues of the CH2 region using genetic engineering
      techniques.  Human Cγ3 genes having a mutation, i.e. 234-leucine to
      alanine, 235-leuciner to glutamine, 236-glycine to alanine, and
      237-glycine to alanine, resp., were constructed and cloned into expression
      vector pSBgpt after linking with the gene encoding the variable domain of
      the B18 **antibody** (Ab).  The binding affinity I50 (the concentration of
      IgG3 at which the fractional binding of 125I-labeled pooled human IgG is
      0.5) to Fc γ R1 receptor on U937 cells of the recombinant mutants,
      i.e. [234-Ala]-IgG3, [235-Glu]-IgG3, [236-Ala]-IgG3, and [237-Ala]-IgG3
      were 4 + 10-8, >10-6, 3 + 10-8, and 3 + 10-7M, resp.,
      vs. 10-8M of the control using the wild-type IgG.

L14  ANSWER 4 OF 4     MEDLINE on STN .
89078461.    PubMed ID: 3060362.  **Complement** activation is not
      required for IgG-mediated suppression of the **antibody** response.
      Heyman B; Wiersma E; Nose M. (Department of Immunology, Uppsala
      University, Sweden. ) European journal of immunology, (1988 Nov) Vol. 18,
      No. 11, pp. 1739-43.  Journal code: 1273201. ISSN: 0014-2980. Pub.
      country: GERMANY, WEST: Germany, Federal Republic of. Language: English.
AB    Feedback suppression of the **antibody** response by IgG is known to
      be dependent on intact Fc regions.  However, it is not clear which of the
      Fc-mediated **effector functions** is required.  In the
      present report we have studied whether ability or inability of the IgG
      **antibodies** to activate the **complement** system was of.
      consequence for their immunosuppressive effect.  First, a monoclonal
      IgG1-anti-2,4,6-trinitrophenyl (TNP) **antibody**, unable to
      activate **complement** via the classical or alternate pathway, was
      shown to be able to inhibit more than 90% of the in vivo sheep
      erythrocyte-specific **antibody** response in mice when TNP coupled
      to sheep erythrocytes was used as antigen.  Second, we investigated the
      immunosuppressive ability of a non-**complement**-activating mutant
      IgG2a-anti-TNP monoclonal **antibody**.  The mutant differs from the
      wild type by a single amino acid **substitution** in the **CH2**
      **domain** leading to inability to fix **complement** factor
      C1q.  However, the mutant has the same affinity for antigen and the same
      Fc receptor-binding capacity as the wild type **antibody**.  It is
      demonstrated that the mutant was as efficient as the wild type
      **antibody** in inhibiting an in vitro **antibody** response to
      TNP-coupled sheep erythrocytes.  These findings confirm the

non-determinant specificity and Fc dependence of IgG-mediated suppression, and show that the Fc-mediated effector mechanism is independent of **complement** activation.  The results instead suggest binding to Fc receptors as a necessary step in feedback immunosuppression and favor inactivation of B cells by cross-linking of Fc and antigen receptors on their surface rather than elimination of antigen by **complement** -dependent phagocytosis as the effector mechanism.


```
=> s l12 and chimeric
L15            8 L12 AND CHIMERIC

=> dup remove l15
PROCESSING COMPLETED FOR L15
L16             8 DUP REMOVE L15 (0 DUPLICATES REMOVED)

=> d l16 1-8 cbib abs
```

L16  ANSWER 1 OF 8    MEDLINE on STN
2007454199.    PubMed ID: 17571902.    Fc glycans terminated with
    N-acetylglucosamine residues increase **antibody** resistance to
    papain. Raju T Shantha; Scallon Bernard. (Discovery Research, Centocor R&D
    Inc., 145 King of Prussia Road, Radnor, Pennsylvania 19087. )
    Biotechnology progress, (2007 Jul-Aug) Vol. 23, No. 4, pp. 964-71.
    Electronic Publication: 2007-06-16. Journal code: 8506292. ISSN:
    8756-7938. Pub. country: United States. Language: English.
AB   Glycosylation in the **CH2 domain** of Fc is required for
    immunoglobulins G (IgGs) to exhibit immune **effector**
    **functions** including **complement**-dependent cytotoxicity
    (CDC) and **antibody**-dependent (Ab-dependent) cellular
    cytotoxicity (ADCC).  We recently established that glycosylated Abs are
    more resistant to papain digestion than non-glycosylated IgGs (Biochem.
    Biophys.  Res.  Commun. 2006, 341, 797-803).  To test whether specific Fc
    glycan structures affect Ab resistance to papain, we used in vitro
    glycoengineering methods to prepare homogeneous Ab glycoforms terminated
    with either sialic acid (G2S2), beta-galactose (G2), or
    N-acetylglucosamine (G0) and subjected them to papain digestions.
    Analyses of aliquots taken at different times during the digestions by
    matrix-assisted laser desorption-time-of-flight-mass spectroscopy
    (MALDI-TOF-MS) and high-performance liquid chromatography (HPLC) methods
    showed that the G0 glycoform was at least two times more resistant to
    papain digestion than the G2 and G2S2 glycoforms.  The increased
    resistance of the G0 glycoform over the G2 and G2S2 glycoforms was
    independent of the specific Ab analyzed.  A mouse/human **chimeric**
    version of Ab1, a fully human version of Ab2, and a humanized version of
    Ab3 exhibited a similar pattern of glycoform-dependent resistance.  These
    data suggest that terminal sugars of Fc glycans may play important roles
    in Ab stability and affect resistance to proteases in addition to
    impacting Ab **effector functions.**

L16  ANSWER 2 OF 8  EMBASE  COPYRIGHT (c) 2007 Elsevier B.V. All rights
    reserved on STN
2004128302  EMBASE  The Evaluation of Recombinant, **Chimeric**,
    Tetravalent Antihuman CD22 **Antibodies.** Meng R.; Smallshaw J.E.;
    Pop L.M.; Yen M.; Liu X.; Le L.; Ghetie M.-A.; Vitetta E.S.; Ghetie V..
    E.S. Vitetta, Cancer Immunobiology Center, Univ. of TX Southwestern Med.
    Ctr., 6000 Harry Hines Boulevard, Dallas, TX 75390-8576, United States.
    ellen.vitetta@utsouthwestern.edu. Clinical Cancer Research  Vol. 10, No.
    4, pp. 1274-1281  15 Feb 2004.
    Refs: 21.
    ISSN: 1078-0432. CODEN: CCREF4
    Pub. Country: United States. Language: English. Summary Language: English.
    Entered STN: 20040415. Last Updated on STN: 20040415
AB   Purpose: The purpose of this study was to prepare **chimeric**
    antihuman CD22 tetravalent monoclonal **antibodies** (MAbs) with

high functional affinity, long persistence in the circulation, increased
antitumor activity, and conserved **effector function** in
vitro. Experimental Design: We investigated the association/ dissociation
rates of these tetravalent **antibodies** using CD22 (+) Daudi
lymphoma cells. We then tested their ability to interact with Fc
receptors on a human cell line (U937), to mediate **antibody**
-dependent cellular cytotoxicity with human natural killer cells, to bind
human C1q, to inhibit the in vitro growth of CD22 Daudi cells, and to
persist in the circulation. Results: The rate of dissociation of the
tetravalent MAbs versus the divalent **antibody** was considerably
slower. These tetravalent MAbs inhibited the in vitro proliferation of
CD22 Daudi cells at a concentration that was at least 100-fold lower than
that of the divalent murine **antibody.** The tetravalent MAbs
containing both the CH2 and CH3 domains and a **chimeric**
recombinant divalent **antibody** bound similarly to Fc receptor,
C1q, and mediate **antibody**-dependent cellular cytotoxicity
equally well with human natural killer cells. The persistence in the
circulation of **chimeric** tetravalent MAbs was considerably longer
than that of chemical homodimers. Conclusions: The tetravalent anti-CD22
MAbs with intact Fc regions should make effective therapeutic agents for
B-cell tumors.

L16    ANSWER 3 OF 8   SCISEARCH   COPYRIGHT (c) 2007 The Thomson Corporation   on
       STN
1998:778085    The Genuine Article (R) Number: 127GV. Therapy with monoclonal
       **antibodies.** II. The contribution of Fc gamma receptor binding and
       the influence of C(H)1 and C(H)3 domains on in vivo **effector**
       **function.** Isaacs J D (Reprint); Greenwood J; Waldmann H. St James
       Univ Hosp, Mol Med Unit, Clin Sci Bldg, Leeds LS9 7TF, W Yorkshire,
       England (Reprint); Univ Cambridge, Dept Pathol, Div Immunol, Cambridge CB2
       1QP, England. JOURNAL OF IMMUNOLOGY (15 OCT 1998) Vol. 161, No. 8, pp.
       3862-3869. ISSN: 0022-1767. Publisher: AMER ASSOC IMMUNOLOGISTS, 9650
       ROCKVILLE PIKE, BETHESDA, MD 20814 USA. Language: English.
       *ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS*
AB         An in vivo model is used to define Fe motifs engaged bg mAbs to
       deplete target cells. Human IgG1 and human IgG4 were very patent, and
       mutations within a motif critical for Fc gamma R binding (glutamate 233 to
       proline, leucine/phenylalanine 234 to valine, and leucine 235 to alanine)
       completely prevented depletion, Mouse IgG2b was also potent, and mutations
       to prevent **complement** activation did not impair depletion with
       this isotype, as previously shown for human IgG1. In contrast, a mutation
       that impaired binding to mouse Fc gamma RII (glutamate 318 to alanine)
       eliminated **effector function** of mouse IgG2b and also
       reduced the potency of human IgG4. To reveal potential contributions of
       domains other than C(H)2, domain switch mutants were created between human
       IgG1 and rat IgG2a, Two hybrid mAbs were generated with potencies
       exceeding anything previously seen in this model, While their mechanism of
       depletion was not defined, their activity appeared dependent upon
       interdomain interactions in the Pc region.

L16    ANSWER 4 OF 8   SCISEARCH   COPYRIGHT (c) 2007 The Thomson Corporation   on
       STN
1995:126358    The Genuine Article (R) Number: QH291. ADDITION OF A MU-TAILPIECE
       TO IGG RESULTS IN POLYMERIC **ANTIBODIES** WITH ENHANCED
       **EFFECTOR FUNCTIONS** INCLUDING **COMPLEMENT**
       -MEDIATED CYTOLYSIS BY IGG4. SMITH R I F (Reprint); COLOMA M J; MORRISON
       S L. UNIV CALIF LOS ANGELES, DEPT MICROBIOL & MOLEC GENET, LOS ANGELES, CA
       90024; UNIV CALIF LOS ANGELES, INST MOLEC BIOL, LOS ANGELES, CA 90024.
       JOURNAL OF IMMUNOLOGY (1 MAR 1995) Vol. 154, No. 5, pp. 2226-2236. ISSN:
       0022-1767. Publisher: AMER ASSOC IMMUNOLOGISTS, 9650 ROCKVILLE PIKE,
       BETHESDA, MD 20814. Language: English.
       *ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS*
AB         The 18-amino acid carboxyl-terminal tailpiece from IgM (mu tp) has
       now been added to the carboxyl-termini of IgG1, IgG2, IgG3, and IgG4
       constant regions to produce recombinant IgM-like IgGs. Polymeric IgCs

obtained by this approach possess up to six Fcs and 12 antigen-combining
sites, greatly increasing the avidity of their interactions with other
molecules. Not surprisingly, the C activity of normally active IgG1 and
IgG3 and somewhat less active IgG2 Abs is shown to be dramatically
enhanced upon polymerization. The multiple Fcs present in a single
molecule apparently allow for more efficient interactions with the
multiple C1q heads present in C1, the first component of the classical C
cascade. An unexpected result however, is that IgG4, normally devoid of C
activity, when polymerized in the same fashion directs C-mediated lysis of
target cells almost as effectively as the other polymers. Interestingly
though, IgG4 mu tp does not deplete C activity in a standard consumption
assay using soluble Ag. The other gamma mu tp isotypes are capable of
depleting 100% of the serum lytic ability even in the absence of Ag,
whereas IgG4 mu tp shows no evidence of activity in this assay under any
of the conditions tested. Additionally, we show that, in contrast to
monomeric IgG, polymeric IgCs bind with very high affinity to Fc gamma
receptor II (Fc gamma RII), a low affinity receptor for wild-type
**antibodies**; however, binding to Fc gamma RI, the high affinity
receptor, appears to be unaltered. Finally, the in vivo t(1/2) of the
gamma mu tp proteins is decreased relative to wild-type IgG, apparently
because of rapid clearance of the polymeric fraction.

L16   ANSWER 5 OF 8  SCISEARCH  COPYRIGHT (c) 2007 The Thomson Corporation  on
      STN
1994:70790   The Genuine Article (R) Number: MV631. RESIDUE AT POSITION-331 IN
      THE IGG1 AND IGG4 **CH2 DOMAINS** CONTRIBUTES TO THEIR
      DIFFERENTIAL ABILITY TO BIND AND ACTIVATE **COMPLEMENT.**  XU Y
      (Reprint); OOMEN R; KLEIN M H. UNIV TORONTO, DEPT IMMUNOL, MED SCI BLDG,
      TORONTO M5S 1A8, ONTARIO, CANADA; CONNAUGHT CTR BIOTECHNOL RES, N YORK M2R
      3T4, ON, CANADA; UNIV TORONTO, DEPT BIOCHEM, TORONTO M5S 1A8, ONTARIO,
      CANADA. JOURNAL OF BIOLOGICAL CHEMISTRY (4 FEB 1994) Vol. 269, No. 5, pp.
      3469-3474. ISSN: 0021-9258. Publisher: AMER SOC BIOCHEMISTRY MOLECULAR
      BIOLOGY INC, 9650 ROCKVILLE PIKE, BETHESDA, MD 20814. Language: English.
      *ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS*
AB         A conserved proline residue is found at position 331 in the C(H)2
      domains of human IgG subclasses which fix **complement.** This
      residue is replaced by a serine in IgG4 which is inactive. To determine
      the role of residue 331 in the differential ability of human IgGs to
      activate the **complement** cascade, a pair of genetically
      engineered anti-dinitrophenol IgG1 and IgG4 **antibodies** with
      reciprocal mutations at position 331 were tested for their hemolytic
      activity as well as for their ability to bind C1q, activate C1 and cleave
      C4. The IgG1 Ser331 mutant was virtually unable to mediate the lysis of
      trinitrobenzene-sulfonic acid-derivatized sheep red blood cells as a
      result of a marked defect in C1q binding activity. In contrast, the
      substitution of Pro for Ser331 in IgG4 bestowed partial hemolytic activity
      (40%) to the IgG4 Pro331 variant. Under low ionic strength conditions,
      this mutant was found to be approximately 50 and 75% as active as
      wild-type IgG1 in the C1q binding and C4b deposition assays, respectively.
      These results indicate that residue Pro''', which folds into close
      proximity to a previously identified C1q binding motif (Duncan, A.  R.,
      and Winter, G. (1988) Nature 332, 738-740), contributes to the
      architecture of the IgG1 C1q binding site and that its replacement by a
      serine residue in IgG4 is largely responsible for the functional
      inactivity of this isotype.

L16   ANSWER 6 OF 8  SCISEARCH  COPYRIGHT (c) 2007 The Thomson Corporation  on
      STN
1993:474217   The Genuine Article (R) Number: LP830. STRUCTURAL FEATURES OF
      HUMAN IMMUNOGLOBULIN-G THAT DETERMINE ISOTYPE-SPECIFIC DIFFERENCES IN
      **COMPLEMENT** ACTIVATION.  TAO M H (Reprint); SMITH R I F; MORRISON S
      L. UNIV CALIF LOS ANGELES, INST MOLEC BIOL, LOS ANGELES, CA 90024; UNIV
      CALIF LOS ANGELES, DEPT MICROBIOL & MOLEC GENET, LOS ANGELES, CA 90024.
      JOURNAL OF EXPERIMENTAL MEDICINE (1 AUG 1993) Vol. 178, No. 2, pp. 661-667
      . ISSN: 0022-1007. Publisher: ROCKEFELLER UNIV PRESS, 1114 FIRST AVE, 4TH

AB       Although very similar in sequence, the four subclasses of human
immunoglobulin G (IgG) differ markedly in their ability to activate
**complement.** Glu318-Lys320-Lys322 has been identified as a key
binding motif for the first component of **complement**, C1q, and is
present in all isotypes of Ig capable of activating **complement.**
This motif, however, is present in all subclasses of human IgG, including
those that show little (IgG2) or even no (IgG4) **complement**
activity. Using point mutants of **chimeric antibodies**,
we have identified specific residues responsible for the differing ability
of the IgG subclasses to fix **complement.** In particular, we show
that Ser at position 331 in gamma4 is critical for determining the
inability of that isotype to bind C1q and activate **complement.**
Additionally, we provide further evidence that levels of C1q binding do
not necessarily correlate with levels of **complement** activity,
and that C1q binding alone is not sufficient for **complement**
activation.

L16   ANSWER 7 OF 8   SCISEARCH   COPYRIGHT (c) 2007 The Thomson Corporation   on
STN
1991:186060    The Genuine Article (R) Number: FE128. THE DIFFERENTIAL ABILITY
OF HUMAN IGG1 AND IGG4 TO ACTIVATE **COMPLEMENT** IS DETERMINED BY
THE COOH-TERMINAL SEQUENCE OF THE **CH2 DOMAIN.** TAO M H
(Reprint); CANFIELD S M; MORRISON S L. UNIV CALIF LOS ANGELES, DEPT
MICROBIOL & MOLEC GENET, LOS ANGELES, CA 90024; COLUMBIA UNIV COLL PHYS &
SURG, DEPT MICROBIOL, NEW YORK, NY 10032. JOURNAL OF EXPERIMENTAL MEDICINE
(1 APR 1991) Vol. 173, No. 4, pp. 1025-1028. ISSN: 0022-1007. Publisher:
ROCKEFELLER UNIV PRESS, 222 E 70TH STREET, NEW YORK, NY 10021. Language:
English.
AB       Using domain switch **chimeric antibodies**, we
confirm the important role of C(H)2 in **complement** activation.
In addition, we demonstrate that the structures responsible for the
differential ability of human IgG1 and IgG4 to activate **complement**
are located at the COOH-terminal part (from residue 292 to 340) of the
C(H)2 domain. The amino acids in C(H)2 that might be involved in
**complement** interaction are discussed. While C(H)3 contributes to
efficient **complement** activation, C(H)3 from IgG2 and C(H)3 IgG3
are equally effective.

L16   ANSWER 8 OF 8   CAPLUS   COPYRIGHT 2007 ACS on STN
1989:613045    Document No. 111:213045   Studies of aglycosylated
**chimeric** mouse-human IgG. Role of carbohydrate in the structure
and **effector functions** mediated by the human IgG
constant region. Tao, Mi Hua; Morrison, Sherie L. (Coll. Physicians
Surg., Columbia Univ., New York, NY, 10032, USA). Journal of Immunology,
143(8), 2595-601 (English) 1989. CODEN: JOIMA3. ISSN: 0022-1767.
AB   **Chimeric** mouse-human IgG was used to study the structural and
functional roles of the carbohydrate present in the **CH2**
**domain** of human IgG mols. To remove this N-linked carbohydrate,
Asn-297, the oligosaccharide attachment residue, was changed to either Gln
(a conservative replacement) or His for IgG1 or Lys for IgG3
(nonconservative replacements) by site-directed mutagenesis.
Carbohydrate-deficient **antibodies** are properly assembled and
secreted and bind Ag and protein A. However, aglycosylated IgG are more
sensitive to most proteases than their corresponding wild-type IgG,
indicating some conformational changes have occurred. Aglycosylated IgG
do not bind to the human Fc$\gamma$RI and do not activate C; depending on
the isotype, **complement** C1q binding ability is either completely
lost (IgG1) or dramatically decreased (GG). The serum half-life in mice
of aglycosylate IgG1-Gln remains the same as wild-type IgG1, whereas
aglycosylated IgG3-Gln has a shorter half-life, compared to that of
wild-type IgG3. Thus, the carbohydrate interposed between **CH2**
**domain** of human IgG is necessary to maintain the appropriate

structure for the maintenance of many of the **effector
functions** dependent on the **CH2 domain.**


=> s (armour k?/au or clark m?/au or williamson l?/au)
L17      14607 (ARMOUR K?/AU OR CLARK M?/AU OR WILLIAMSON L?/AU)

=> s l17 and chimeric CH2 domain
L18         0 L17 AND CHIMERIC CH2 DOMAIN

=> s l17 and CH2 domain
L19        22 L17 AND CH2 DOMAIN

=> dup remove l19
PROCESSING COMPLETED FOR L19
L20         6 DUP REMOVE L19 (16 DUPLICATES REMOVED)

=> d l20 1-6 cbib abs

L20  ANSWER 1 OF 6     MEDLINE on STN                   DUPLICATE 1
2003519950.    PubMed ID: 14597161.    Differential binding to human FcgammaRIIa
     and FcgammaRIIb receptors by human IgG wildtype and mutant antibodies.
     **Armour Kathryn L;** van de Winkel Jan G J; **Williamson Lorna
     M; Clark Mike R.** (Department of Pathology, Division of
     Immunology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP,
     UK. ) Molecular immunology, (2003 Dec) Vol. 40, No. 9, pp. 585-93.
     Journal code: 7905289. ISSN: 0161-5890. Pub. country: England: United
     Kingdom. Language: English.
AB   We are investigating the interactions of recombinant human IgG antibodies
     with Fc receptors to enable selection of a constant region giving minimal
     depletion of antigen-bearing cells.  Eight variant constant regions were
     made by substituting motifs between human IgG subclasses in the lower
     hinge region and/or a specially close loop of the **CH2
     domain.**  Mutations in the lower hinge region were shown to
     eliminate FcgammaRI binding and monocyte activation [Eur.  J.  Immunol. 29
     (1999) 2613].  Here, we detail interactions with FcgammaRIIa of the 131R
     and 131H allotypes and FcgammaRIIb.  Lower hinge mutations caused large
     reductions in binding whereas modification of residues 327, 330 and 331
     had less dramatic effects.  However, like the wildtype IgG subclass
     binding hierarchies, the effect of the mutations varied between different
     receptors.  We identified IgG1 variants which react with the activating
     receptor, FcgammaRIIa, at least 10-fold less efficiently than wildtype
     IgG1 but whose binding to the inhibitory receptor, FcgammaRIIb, is only
     four-fold reduced.  Manipulation of interactions with FcgammaRIIb
     separately from those with activating receptors provides potential for
     designing antibodies with novel and effective combinations of attributes.
     In addition, insight is gained into the evolution of functional
     differences in human IgG subclasses.

L20  ANSWER 2 OF 6     MEDLINE on STN                   DUPLICATE 2
2002438623.    PubMed ID: 12196122.    The contrasting IgG-binding interactions
     of human and herpes simplex virus Fc receptors. **Armour K L;**
     Atherton A; **Williamson L M; Clark M R.** (Department of
     Pathology, University of Cambridge, Cambridge, UK..
     kla22@mole.bio.cam.ac.uk) . Biochemical Society transactions, (2002 Aug)
     Vol. 30, No. 4, pp. 495-500.  Ref: 37. Journal code: 7506897. ISSN:
     0300-5127. Pub. country: England: United Kingdom. Language: English.
AB   A virally encoded, high-affinity Fc receptor (FcR) is found on herpes
     simplex virus type 1 (HSV-1) particles and infected cells where its
     binding of non-immune IgG protects cells from host-mediated lysis.  Whilst
     mutation or aglycosylation of the IgG **CH2 domain**
     reduced binding to human FcR, the interaction with HSV-1 FcR was not
     affected.  However, the HSV-1 FcR, unlike human FcR, discriminates between
     human IgG1 allotypes, being sensitive to changes at positions 214 (CH1)
     and 356/358 (CH3), away from its proposed binding site at the CH2-CH3

interface.  The biological consequences are not known but this is the
first evidence of a major functional difference between IgG1 allotypes.

L20   ANSWER 3 OF 6   CAPLUS   COPYRIGHT 2007 ACS on STN
1999:736770   Document No. 131:350263   Chimeric proteins containing IgG Fc
    fragments which do not trigger complement mediated lysis.  **Armour,
    Kathryn Lesley; Clark, Michael Ronald; Williamson,
    Lorna McLeod** (Cambridge University Technical Services Limited, UK).
    PCT Int. Appl. WO 9958572 A1 19991118, 81 pp.  DESIGNATED STATES: W:  AE,
    AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE,
    ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR,
    KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,
    RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU,
    ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG,
    CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR,
    NE, NL, PT, SE, SN, TD, TG.  (English).  CODEN: PIXXD2.  APPLICATION: WO
    1999-GB1441 19990507.  PRIORITY: GB 1998-9951 19980508.
AB    The authors disclose recombinant polypeptides comprising: (i) a binding
    domain capable of binding a target mol., and (ii) an effector domain
    having an amino acid sequence substantially homologous to all or part of a
    constant domain of a human Ig heavy chain.  These chimeric proteins are
    capable of binding the target mol. without triggering significant
    complement dependent lysis, or cell mediated destruction of the target
    and, via the effector domain, remain capable of specifically binding FcRn
    and/or FcγRIIb.  These effector domains are derived from two or more
    human Ig heavy chain **CH2 domains.**  The binding domain
    of the chimeric proteins may be derived from antibodies, enzymes,
    hormones, receptors, and cytokines etc.

L20   ANSWER 4 OF 6      MEDLINE on STN                    DUPLICATE 3
1999388014.     PubMed ID: 10458776.    Recombinant human IgG molecules lacking
    Fcgamma receptor I binding and monocyte triggering activities. **Armour
    K L; Clark M R;** Hadley A G; **Williamson L M.**
    (Division of Immunology Department of Pathology, University of Cambridge,
    Cambridge, GB. ) European journal of immunology, (1999 Aug) Vol. 29, No.
    8, pp. 2613-24.  Journal code: 1273201. ISSN: 0014-2980. Pub. country:
    GERMANY: Germany, Federal Republic of. Language: English.
AB    Subclasses of human IgG have a range of activity levels with different
    effector systems but each triggers at least one mechanism of cell
    destruction.  We are aiming to engineer non-destructive human IgG constant
    regions for therapeutic applications where depletion of cells bearing the
    target antigen is undesirable.  The attributes required are a lack of
    killing via Fcgamma receptors (R) and complement but retention of neonatal
    FcR binding to maintain placental transport and the prolonged half-life of
    IgG.  Eight variants of human IgG constant regions were made with anti-RhD
    and CD52 specificities.  The mutations, in one or two key regions of the
    **CH2 domain,** were restricted to incorporation of motifs
    from other subclasses to minimize potential immunogenicity.  IgG2 residues
    at positions 233 - 236, substituted into IgG1 and IgG4, reduced binding to
    FcgammaRI by 10(4)-fold and eliminated the human monocyte response to
    antibody-sensitized red blood cells, resulting in antibodies which blocked
    the functions of active antibodies.  If glycine 236, which is deleted in
    IgG2, was restored to the IgG1 and IgG4 mutants, low levels of activity
    were observed.  Introduction of the IgG4 residues at positions 327, 330
    and 331 of IgG1 and IgG2 had no effect on FcgammaRI binding but caused a
    small decrease in monocyte triggering.

L20   ANSWER 5 OF 6   SCISEARCH   COPYRIGHT (c) 2007 The Thomson Corporation   on
    STN
1995:111533   The Genuine Article (R) Number: QG208. INTERACTION OF HUMAN
    MONOCYTE FC-GAMMA RECEPTORS WITH RAT IGG2B - A NEW INDICATOR FOR THE
    FC-GAMMA-RIIA (R-H131) POLYMORPHISM.  HAAGEN I A (Reprint); GEERARS A J G;
    **CLARK M R;** VANDEWINKEL J G J. UNIV UTRECHT HOSP, DEPT IMMUNOL
    F03821, POSTBOX 85500, 3508 GA UTRECHT, NETHERLANDS (Reprint); UNIV
    CAMBRIDGE, DEPT PATHOL, CAMBRIDGE, ENGLAND. JOURNAL OF IMMUNOLOGY (15 FEB

*ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS*

AB      Rat mAbs receive considerable interest for immunologic intervention
in man.  The rat IgG2b isotype has previously been found to be optimally
active both in vivo and in vitro.  We found that both a rat IgG2b CD3 mAb
and a monovalent hybrid rat IgG2b-mouse IgG1 bispecific Ab triggered T
cell activation in PBMC.  Inhibition analyses with mAb blocking different
human IgG Fc receptors (Fc gamma R) showed a dimorphic pattern.  In donors
expressing an Fc gamma RIIa-R/R131 allotype (previously defined on the
basis of interaction with mouse (m) IgG1 as ''high responder'') anti-Fc
gamma RI mAb 197 inhibited rat IgG2b induced T cell mitogenesis almost
completely.  In Fc gamma RIIa-H/H131 (''low responder'' allotype) donors,
however, both anti-Fc gamma RI mAb 197 and anti-Fc gamma RII mAb IV.3 were
essential for optimal inhibition of mitogenesis.  T cell proliferation
experiments performed with the use of Fc gamma R-transfected fibroblasts
as accessory cells showed the high affinity Fc gamma RIa (CD64) to
interact with both rat IgG2b and rat IgG2b-mIgG1 hybrid CD3 mAb.  The use
of the two types of Fc gamma RIIa (CD32)-transfectants instead showed rat
IgG2b CD3 mAb to interact solely with the IIa-H/H131 allotype.
Interestingly, rat IgG2b-mIgG1 hybrid mAb did not interact effectively
with this low affinity Fc gamma R.  This suggests a requirement for only
one rat IgG2b H chain for Fc gamma RIa-mediated binding, whereas two
identical H chains seem to be necessary for proper interaction with Fc
gamma RIIa.  Ab-sensitized RBC-rosette experiments performed with the use
of a rat IgG2b anti-NIP mAb confirmed the interaction pattern observed
with rat CD3 mAb, supporting the phenomena to be isotype-, and not mAb-,
dependent.  These analyses point to a unique reactivity pattern for rat
IgG2b Abs, interacting both with the high affinity Fc gamma RIa in all
donors and Fc gamma RIIa of individuals expressing the IIa-H131 allotype.

L20  ANSWER 6 OF 6     MEDLINE on STN                    DUPLICATE 4
AB   A humanized IgG antibody to CAMPATH-1 antigen (CDw52) is known to be
lympholytic both in vitro and in vivo.  So as to improve therapeutic
potency through protein engineering strategies, we wish to define the
structural motifs underlying some of the documented differences in
function between human (h) IgG1 and IgG4 forms of the antibody.  By the
creation of heavy chain domain-switch and intra-domain recombinant
antibodies we have established an important role for the carboxy-terminal
half of the **CH2 domain** in determining differential
behaviour in antibody-dependent cytotoxicity (ADCC) and in complement
lysis.  If this same region were necessary for the effector mechanisms
that operate in vivo, then it might be possible to improve antibody
effector functions by construction of novel antibodies that possess within
the one molecule multiple copies of the crucial hinge-CH2 associated
structures.  Although our previous work suggested that the hIgG4 CAMPATH-1
antibody was ineffective at ADCC, we found this to be so only in some
individuals.  In others, IgG4, and indeed all the IgG subclasses were able
to mediate ADCC.  Overall, though, hIgG1 remains the best choice isotype
for lytic therapy in vivo.


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---Logging off of STN---

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Executing the logoff script...


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FULL ESTIMATED COST                                   144.30        144.51

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